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# **Describing the Gut Microbiome and Metabolomic Changes in Bile Acid Diarrhoea (BAD)**

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A thesis submitted in partial fulfilment of the requirements for the  
degree of Doctor of Medicine

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For my son Aaryn  
and  
in memory of my father Dr Gian Sagar.

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## **Abbreviations**

°C	degrees centigrade
cms	centimetres
g	grams
kb	kilobyte
L	litre
M	molar
mg	milligrams
mL	millilitres
mM	millimolar
mmol	millimole
m/s	metres per second
ng	nanograms
pg	pictogram
pM	particulate matter
µg	microgram
µl	microlitre
µM	micrometre/micron
µmol	micromole
V	volt
5HT <sub>3</sub>	5 hydroxytryptamine (subtype 3 serotonin receptor)
A.	Akkermansia
ACTH	Adrenocorticotropic hormone
AJC	Apical junction complex
AMP	Adenosine monophosphate
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
ASBT	Apical sodium dependent bile acid transporter
ATP	Adenosine triphosphate



AUC	Area under the curve
<i>B.</i>	Bifidobacterium
BA	Bile acids
BAD	Bile acid diarrhoea
BAS	Bile acid sequestrants
BD	Bis in die (twice daily)
BMI	Body mass index
BSH	Bile salt hydrolases
C4	7 alpha-hydroxy-4-cholesten-3-one
CA	Cholic acid
Ca <sup>2+</sup>	Calcium
CCK	Cholecystokinin
CD	Cluster of differentiation
CDCA	Chenodeoxycholic acid
CFU	Colony forming units
Cg	Chromatogranins
CH <sub>4</sub>	Methane
CI	Confidence intervals
CINC	Cytokine-induced neutrophil chemoattractant
Cl <sup>-</sup> /OH <sup>-</sup>	Chloride hydroxide anion
CLDN	Claudins
CNS	Central nervous system
CO <sub>2</sub>	Carbon dioxide
CRF	Corticotrophin-releasing factor
CRP	C-reactive protein
CYP7A1	Cholesterol 7 alpha-hydroxylase
CTP8B1	Microsomal sterol 12 alpha-hydroxylase
DC	Dendritic cell
DCA	Deoxycholic acid
DGCE	Denaturing gradient gel electrophoresis
dH <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic acid
DSS	Dextran sulphate sodium
EGFR	Epithelial growth factor receptor

ENS	Enteric nervous system
ERK 1/2	Extracellular signal-regulated kinase 1/2
FAIMS	Field asymmetric ion mobility spectrometry
FBD	Functional bowel disorder
FCP	Faecal calprotectin
FGF-15	Fibroblast growth factor 15
FGF-19	Fibroblast growth factor 19
FGFR4	Fibroblast growth factor receptor 4
FISH	Fluorescence in situ hybridisation
FMT	Faecal microbiota transplant
FODMAPS	Fermentable, oligo-, di-, monosaccharides and polyols
FOS	Fructo-oligosaccharides
FXR	Farnesoid X receptor
GC-MS	Gas chromatography mass spectrometry
GI	Gastrointestinal
GPBAR1	G protein-coupled bile acid receptor 1
GPCR	G protein-coupled receptor
H <sub>2</sub>	Hydrogen
HCS	Healthy controls
HPA	Hypothalamic-pituitary-axis
HSDS	Homoserine dehydrogenase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-C	Constipation- predominant irritable bowel syndrome
IBS-D	Diarrhoea-predominant irritable bowel syndrome
IBS-M	Mixed irritable bowel syndrome
IBS-U	Unclassified irritable bowel syndrome
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
JNK 1/2	c-Jun N-terminal kinase 1/2
KLKB1	Klotho $\beta$
L.	Lactobacillus

LCA	Lithocholic acid
LDA	Linear discriminant analysis
LDL	Low-density lipoprotein
LFD	Low FODMAPS diet
LPL	Lipoprotein lipase
MCP	Monocyte chemoattractant protein
MLC	Myosin light chain
mRNA	Messenger ribonucleic acid
N	Normality
NADH	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
NHS	National Health Service
NICE	National institute of clinical excellence
NO	Nitric oxide
NPV	Negative predictive value
OCLN	Occludins
OTU	Operational taxonomic uni
PCA	Principal component analysis
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PI <sub>3</sub>	Phosphoinositide 3-kinase
PPV	Positive predictive value
<i>R.</i>	Ruminococcus
RCT	Randomised controlled trial
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
ROC	Receiver operator curve
rpm	Revolutions per minute
SCFA	Short-chain fatty acid
SD	Standard deviation
Sec	Second
SeHCAT	<sup>75</sup> Selenium homotaurocholic acid test
Sg	Secretogranins
SHP	Small heterodimer partner

SO <sub>4</sub>	Sulphate
spp	Species
sprr2a	Small proline-rich protein 2a
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
TtG	Tissue transglutaminase
TRFP	Terminal restriction fragment length polymorphism
UDCA	Ursodeoxycholic acid
UHCW	University Hospitals Coventry and Warwickshire
UK	United Kingdom
V3-V4	Variable 3-variable 4 gene fragment
VOCs	Volatile organic compounds
WMS	Whole metagenomic shotgun

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## **Declaration**

I, Nidhi Sagar, declare that this thesis has not been submitted for a degree at another University.

The presented work, including data processing and data analysis, was carried out by the author except in the cases outlined below:

- Parts of the bioinformatics analysis (QIIME input undertaken by the author) performed by Dr Christopher Quince (chapter 2).
- Measurement of short-chain fatty acids (SCFAs) by gas chromatography performed by Dr Kostantinos Gerasimidis, Margarita Kokkorou and Vaivos Savlos (chapter 3).
- Measurement of bile acids (BAs) by liquid chromatography-mass spectrometry and statistical analysis performed by Dr Henri Duboc (chapter 4).
- Measurement of volatile organic compounds (VOCs) by FAIMS and statistical analysis performed by Alfian Wicaksono (chapter 5).

## **List of Publications**

1. Sagar NM, Cree IA, Covington JA et al. The interplay of the gut microbiome, bile acids, and volatile organic compounds. *Gastroenterol Res Pract*. 2015; 2015:398585
2. Esfahani S, Sagar NM, Kyrou I et al. Variation in Gas and Volatile Compound Emissions from Human Urine as It Ages, Measured by an Electronic Nose. *Biosensors (Basel)*. 2016 Jan 25; 6(1). (Joint first authors).
3. Sagar NM, McFarlane M, Nwokolo C et al. Mechanisms of triglyceride metabolism in patients with bile acid diarrhea. *World J Gastroenterol*. 2016 Aug 14; 22(30): 6757-63.

## **Abstract**

The diagnosis of BAD is often missed or misdiagnosed for IBS-D as these conditions present similarly with chronic diarrhoea. The principal hindrance to diagnosis of BAD is limited access to the diagnostic SeHCAT scan. Mechanisms of aetiology underlying BAD have not been fully elucidated and to date, an alternative biomarker for BAD that is more accessible and patient preferable, has yet to make its way into clinical practice.

One of the greatest scientific challenges this decade has been understanding the relationship between the gut microbiome, its functionality and role in human health. BAs are metabolised by the gut microbiota, therefore their role as signalling molecules in regulating intestinal homeostasis is influenced primarily by the gut commensals.

This thesis is the first study to profile the gut microbiome in BAD and investigate the mechanisms of how bacterial metabolic products may influence the development of disease. This was achieved by conducting 16S ribosomal RNA gene analysis, the most important target of study in bacterial ecology. Bacterial metabolites (SCFAs and VOCs) and BAs were measured using gas and liquid chromatography, mass and ion mobility spectrometry.

The results indicate intestinal dysbiosis with reduced bacterial diversity in patients with BAD. A statistically significantly greater total concentration of SCFAs with increases in the concentrations of acetate and propionate were observed in BAD compared to IBS-D. A statistically significant increase in the concentrations of faecal primary BAs and serum CDCA was observed in BAD compared to IBS-D. Separation of VOC profiles was evident between the BAD, IBS-D and HC groups but greatest discrimination was between the IBS-D and HC cohorts.

In conclusion, intestinal dysbiosis with altered fermentation and resultant BA dysmetabolism were observed. The metabolic output of the microbiota rather than abundance of specific bacterial taxa appears to be more important in the aetiology of BAD.

# **Chapter 1:**

# **Introduction**



## **1. Introduction**

Diarrhoea is defined as the abnormal passage of loose or liquid stools more than three times daily and/or a daily stool weight more than 200g/day. It is considered chronic if symptoms have persisted for greater than 4 weeks(1). Chronic diarrhoea is prevalent in 4-5% of the Western population and is a common problem encountered in primary care accounting for nearly 1 in 20 referrals to gastroenterology in secondary care(2, 3).

Diarrhoea-predominant irritable bowel syndrome (IBS-D) is a common cause of chronic diarrhoea. Bile acid diarrhoea (BAD) has found to affect a third of patients previously diagnosed with IBS-D and is often overlooked in the differential diagnosis of chronic diarrhoea(2). This is of great clinical significance given that IBS patients form the largest group of patients seen in a general gastroenterology clinic(4). The diagnostic algorithm for investigating chronic diarrhoea outlined by the British Society of Gastroenterology in 2003 places the consideration of investigating BAD at the bottom of the pathway, once all other organic causes of diarrhoea have been excluded(5). This highlights how BAD is often regarded as a rare phenomenon, even among specialists, with frequently missed opportunities for diagnosis, giving rise to a large undiagnosed population. Consequently, the boundaries between IBS-D and BAD become blurred with little separation of these two distinct disease entities.

### **1.1 Bile Acid Diarrhoea (BAD)**

#### **1.1.1 What is BAD?**

BAD is a common cause of chronic diarrhoea and occurs as sequelae of a defect in the enterohepatic circulation of bile acids (BAs), which is discussed in section 1.1.2.

Three types of BAD are classically recognised and categorized according to their underlying aetiology. In 1967, Alan Hofmann first described the syndrome 'Cholerheic Enteropathy', in patients who had undergone an ileocaecal resection and suffered with diarrhoea due to excessive BAs in the colon(6). Descriptions in the late 1960s and early 70s of patients who experienced diarrhoea with ileal disease of less than 100cms in length and responded to treatment with Cholestyramine, a bile acid binder, further supported this occurrence. Patients with ileal disease in excess of 100cms, experienced greater steatorrhea, secondary to BA deficiency with impaired micelle formation and fat digestion(7, 8). This is now known as Type 1 BAD, which is secondary to ileal disease or resection and is typically a result of Crohn's disease or radiation ileitis. In one study, the prevalence of BAD in patients who had undergone ileal resection for Crohn's disease and were in clinical remission was 97%. 54% of patients with unoperated Crohn's disease in clinical remission were also found to have BAD(2).

Type 2 (primary or idiopathic) is the most common cause of BAD and is demonstrated in the absence of intestinal disease as well as being associated with a histologically normal ileum(9). More recently, a new mechanism for type 2 has been proposed where malabsorption of BAs does not occur in these patients. The discovery of Fibroblast Growth Factor-19 (FGF-19), an ileal bile acid-farnesoid X receptor (FXR) dependent hormone, in suppressing BA biosynthesis, generated further interest in its role in BAD(10). This resulted in the discovery of reduced levels of FGF-19 in patients with BAD(11). Consequently, in patients with reduced levels of FGF-19, hepatocytes are unable to downregulate BA synthesis, resulting in a large BA pool with incomplete BA reabsorption and increased delivery of BAs to the colon, triggering diarrhoea. Other potential mechanisms involved in the development of primary BAD are genetic variants in the Klotho  $\beta$  (KLB) and Fibroblast growth factor receptor 4 (FGFR4) genes as well as upregulation of the membrane bound BA receptor, G protein-coupled BA receptor 1 (GPBAR1)(12). The KLB and FGFR4 genes are involved in feedback regulation of BA synthesis and have been associated with faecal

BA excretion and colonic transit in patients with IBS-D(13). Genetic variation in GPBAR1 has also demonstrated quantitative changes in colonic transit and BA secretion(14).

Type 3 BAD comprises of causes which are not included with types 1 and 2 that may interfere with normal recycling of BAs, small bowel motility, reduced storage capacity and saturation of uptake mechanisms. Causes include post cholecystectomy, small bowel bacterial overgrowth, diabetes mellitus, pancreatitis and coeliac disease(15).

A study of 373 patients diagnosed with BAD demonstrated a significantly greater proportion of patients who had undergone a cholecystectomy (27.4% with BAD vs 13.3% without BAD), terminal ileal resection or right hemicolectomy for Crohn's disease (18.4% vs 1.6%), or terminal ileal resection or right hemicolectomy for other reasons (7.4% vs 1.6%). In those with severe BAD, there were significantly more patients who had a previous right hemicolectomy or terminal ileal resection for Crohn's disease or for other reasons. Types 2 and 3 BAD were found to predominate in patients with mild BAD, whilst type 2 was more prevalent in moderate BAD and type 1 in severe BAD(16).

### **1.1.2 Bile Acids and Pathophysiology of BAD**

BAs constitute 50% of the organic components of bile and are saturated, hydroxylated C<sub>24</sub> cyclopentanophenanthrene sterols(17, 18). They are water-soluble detergent molecules, which facilitate dietary lipid absorption in the small intestine through solubilizing fatty acids and monoglycerides, the lipolysis products of triglycerides, and transport them as mixed micelles. In the micelles, the polar BAs can present the hydrophobic fat molecules to the brush border membrane of the intestine, where they diffuse down their concentration gradients into the intestinal epithelial cells, to enable digestion and absorption(19). The majority of fat absorption takes place in the proximal 100cms of the jejunum(20). The BAs then remain in the intestinal lumen until they are absorbed in the terminal ileum.

### *Enterohepatic Circulation of Bile Acids*

The liver synthesizes the two primary BAs: cholic acid (CA) and chenodeoxycholic acid (CDCA). In liver peroxisomes, BAs are further metabolized by conjugation (*N*-acyl amidation) to glycine or taurine to produce the amphipathic structure that encompasses both hydrophobic (lipid-soluble) and polar (hydrophilic) regions. The amino acid conjugate is polar and hydrophilic while the cholesterol portion of the BA is hydrophobic(21). This conjugation step allows for BAs to remain ionised within the duodenum as ionization renders BAs impermeable to cell membranes through increasing their solubility. This property permits spontaneous formation of micelles by allowing a high concentration of BAs to reach the critical micellar concentration(19).

The BAs are then actively secreted across the canalicular membrane into bile and carried to the gallbladder where they are stored(22). Ingestion of a meal results in the secretion of secretin and cholecystokinin (CCK) by chyme. To increase the volume of bile, biliary duct cells are stimulated by secretin to secrete bicarbonate and water. CCK stimulates gallbladder contraction resulting in bile flowing into the duodenum(17).

The primary BAs activate the farnesoid X receptor (FXR) in the liver, stimulating expression of small heterodimer partner (SHP) to inhibit the action of the homolog-1 liver receptor, which controls the upregulation of Cytochrome P450 family 7 subfamily a member 1 (CYP7A1) - the rate-limiting BA synthesis enzyme(23, 24). Through intestinal FXR activity, BA synthesis is also inhibited via stimulation of the expression of FGF19. To impede BA synthesis, FGF19 binds hepatic fibroblast growth factor-4 (FGF4) and activates c-Jun N-terminal kinase 1/2 (JNK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2)(25). BAs are actively reabsorbed from the ileum and circulated back to the liver via the hepatic portal vein and this process is known as the enterohepatic circulation. This ensures recycling of the majority of synthesized BAs to maintain a functional BA pool. In adults, the BA pool is 2-3 grams in size. With each meal, this pool cycles several times, resulting in the BA secretion of 4-6 grams per meal or 12-18 grams

per day. 0.3 grams of BAs are synthesized each day therefore digestion of lipids occurs predominantly through the use of recycled BAs(26).

Only 1-2% of BAs escape the enterohepatic circulation and undergo biotransformation by the gut microbiota in the large bowel to form the secondary BAs, deoxycholic acid (DCA), and lithocholic acid (LCA)(22). These are then excreted in the faeces (0.3-0.5 grams per day)(27). Transformations by the intestinal microbiota include deconjugation (removal of the amino acid side chain), epimerization (of 3-, 7-, and 12-hydroxy groups), oxidation (removal of H<sub>2</sub>), dehydroxylation (replacement of a hydroxyl group with a hydrogen), and hydroxylation (replacement of a hydrogen with a hydroxyl group)(17). This microbial transformation is a key step and is outlined in further detail below:

*Deconjugation:* Bile salt hydrolases (BSHs) are enzymes, which catalyse the hydrolysis of the C24 N-acyl amide bond of conjugated BAs. BSH enzymes have been purified from various bacteria, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Clostridium perfringens*, *Listeria monocytogenes*, as well as several species of *Lactobacillus* and *Bifidobacterium*(18, 28). With regards to the ecological importance of microbial BSH activity, there are three main hypotheses. Firstly, the liberated amino acids arising from the deconjugation reaction may potentially be used as carbon, nitrogen, and energy sources. Glycine may be metabolized to ammonia and carbon dioxide and taurine to ammonia, carbon dioxide, and sulphate, all of which could then be integrated into bacterial metabolites(17). Secondly, the tensile strength of the membranes may increase or a change in membrane fluidity may affect sensitivity to  $\alpha$ -defensins and other host defense molecules through BSHs facilitating incorporation of cholesterol or bile into bacterial membranes (29, 30). Thirdly, BSHs may play a role in bile tolerance and play a detoxification role, allowing for microbial survival in the gastrointestinal tract in the presence of bile salts(17).

*Oxidation and Epimerisation:* Oxidation and epimerization of the 3-, 7-, and

12-hydroxy groups of BAs in the gastrointestinal tract are catalyzed by homoserine dehydrogenase (HSDHs) enzymes, which are expressed by intestinal bacteria. Epimerization of BA hydroxyl groups generates a stable oxo-bile acid intermediate, necessitates the actions of two stereochemically distinct HSDHs and can be performed by a single species containing both  $\alpha$ - and  $\beta$ - HSDHs or by two species, one possessing an  $\alpha$ -HSDH and the other a  $\beta$ -HSDH. 3- $\alpha$  and 3- $\beta$  HSDH enzymes have been discovered in several bacteria belonging to the *Firmicutes* phylum whereas bacteria capable of intraspecies 3-hydroxy epimerization include *Peptostreptococcus productus*, *C. perfringens*, and *Eggerthella lenta*. 7 $\alpha$ -HSDHs have been identified among members of the *Clostridium*, *Eubacterium*, *Bacteroides*, or *Escherichia* genera. Intraspecies 7-hydroxy epimerization has been observed in species of the *Clostridium*, *Eubacterium*, and *Ruminococcus* genera of bacteria(17).

*Dehydroxylation*: The 7 $\alpha$ -dehydroxylation reaction of primary BAs results in the production of the secondary BAs, which predominate in human faeces. Therefore, this is the most quantitatively significant microbial bile salt transformation. Species of the *Firmicutes* phylum (*Clostridium* and *Eubacterium*) retain 7 $\alpha$ -dehydroxylation activity(17). 7  $\alpha/\beta$  -dehydroxylation is restricted to free BAs, therefore a precondition for this to occur is the removal of glycine/taurine BA conjugates via BSH enzymes(31, 32).

### 1.1.3 Symptoms

Through stimulating colonic secretion and motility, the secretomotor effects of BAs in the colon manifest clinically as the symptoms of BAD. This comprises of water diarrhoea, bloating, faecal urgency and faecal incontinence. These symptoms impact considerably on a patient's lifestyle by affecting activities of daily living and limiting travel or the ability to leave the house(11). Abdominal pain, discomfort and bloating have been found to be more common in patients with mild BAD(16).

The mechanisms of action include activation of intracellular secretory processes, increasing mucosal permeability through the detergent effects of

the BAs, inhibiting  $\text{Cl}^-/\text{OH}^-$  exchange via  $\text{Ca}^{2+}$  and  $\text{PI}_3$  kinase dependent pathways, as well as stimulating mucus secretion(33, 34). Typically, BA concentrations above 3 mmol/L will initiate colonic water secretion by activating adenylate cyclase(35, 36). The colonic secretory effects of water and electrolytes have been shown to be structure-specific and are secondary to the presence of two  $\alpha$  hydroxyl groups at the 3, 7 (CDCA) or the 3, 12 (DCA) positions in the BA molecule(33). These effects have not been demonstrated with CA(37). Sulfation of BAs by intestinal bacteria has appeared to abolish the secretory activity of CDCA and convert it to a non-secretory form(38). Furthermore, BAs in the colon are capable of inducing high amplitude, propulsive contractions, thereby contributing to disordered defecation in these patients(19).

#### **1.1.4 Prevalence**

BAD is often recognised as a rare phenomenon and misdiagnosed for IBS-D which is reflected in a recent systematic review stating that in excess of 28% of patients meeting accepted Rome I, II or III criteria for IBS-D have SeHCAT (tauroselcholic [ $^{75}$ selenium] acid nuclear medicine scan – this is described in section 1.1.5) results consistent with BAD. This effect was observed in all countries where the studies originated from(39). In 2009, 18 studies containing 1223 patients with chronic diarrhoea were systematically reviewed to evaluate the prevalence of BAD. This demonstrated that 32% of patients had BAD as defined by a SeHCAT retention of <10%. Interestingly, the data gathered from these studies collected over a 22-year period (from as early as 1985) found that the trends in the prevalence of undiagnosed patients remained fairly static over time. Variation in the prevalence of the severity of BAD was observed with a 4-13% prevalence range for a SeHCAT retention <5%, 12-65% for retention <10% and 18-53% for retention <15%. With regards to the types of BAD being diagnosed in the UK, 61% of patients have type 1 BAD, 22% have type 2 and 15% have type 3(40).

It has been estimated that 1% of the Western population suffer with BAD(41). Half a million adults in the UK alone who are currently being

treated for IBS-D are expected to have BAD(15). In the UK, 5 new diagnoses of BAD per gastroenterologist per year (1% of new patients) are made. Limited diagnosis of BAD is evident in secondary, specialist care with 22% of gastroenterologists rarely testing for BAD or not at all in patients presenting with chronic diarrhoea(40). Failure or a delay in diagnosis manifests in social embarrassment, loss of employment opportunities and recurrent unnecessary investigations for patients(42).

There are several factors, which may influence why BAD is under-diagnosed. One is that it is frequently not considered in the differential diagnosis of chronic diarrhoea as a functional diagnosis is often made in the absence of alarm symptoms and in the presence of normal blood and endoscopic test results. Even when the diagnosis is considered, many patients may not be referred for a definitive diagnostic test (SeHCAT scan)(43). Only 6% of British gastroenterologists were found to investigate for BAD as first line in patients with chronic diarrhoea and only a third used a definitive diagnostic test to do so(40). There is also national variation in the referral for a definitive diagnostic test with 50% of SeHCAT scan requests originating from only 10 hospitals(40). Other clinician independent factors that may hinder making a diagnosis of BAD include the cost of diagnosis, the therapeutic trial of Colestyramine not tolerated by all patients which some physicians use as a definitive diagnostic test and access to diagnostic facilities(15). The latter is observed in several UK hospitals where fewer than 80 of the 250 departments that are equipped to perform a SeHCAT scan ever do so(40).

#### **1.1.5 Diagnosis**

A definitive diagnosis of BAD is desirable to assess severity of disease and tailor treatment appropriately. The 2008 UK NICE Clinical Practice Guidance on the diagnosis and management of IBS does not mention BAD or propose any guidance on appropriate diagnostic techniques (NICE). In the UK, the SeHCAT test is licensed for use in the investigation of BAD and measurement of BA pool loss(44). The NICE scope, which assesses the use of the SeHCAT scan in investigating BAD, mentions that although there



is no direct comparator for SeHCAT, there remains insufficient evidence to determine whether SeHCAT is a cost-effective diagnostic option(45).

SeHCAT is not widely used in clinical practice and in the US, unlike most of Europe, the SeHCAT scan is unavailable and clinicians depend on reaching a diagnosis of BAD by performing a therapeutic trial of BA sequestrants (BAS)(46). In fact, the American Gastroenterology Association medical position statement on IBS (2002) advises on a trial of Colestyramine for suspected BAD in patients with post-infective and post-cholecystectomy diarrhoea(47). This is inadequate as patients may be non-compliant with BAS due to reports of poor palatability and experience of side effects such as abdominal pain, flatulence and borborygmi thereby halting the attempt at diagnosing BAD(15). Symptoms may also only improve with higher doses of a BAS, which patients may have discontinued prematurely and so the diagnosis of BAD may be missed.

The various diagnostic methods for BAD are outlined below. The SeHCAT scan has superseded other diagnostic approaches in being the test of choice in clinical practice. A recent systematic review identified the SeHCAT scan with the greatest diagnostic yield in identifying BAD in patients with functional bowel disorder (FBD) with diarrhoea. Approximately 31% of patients with FBD with diarrhoea had BAD based on a SeHCAT scan result compared to an average of 23% diagnosed by other diagnostic tests, including serum C4, serum FGF19 and total faecal BA excretion(48).

#### *<sup>14</sup>C-glycocholate (<sup>14</sup>C-BA) breath and stool test*

This is a method to determine bacterial-dependent deconjugation due to bacterial overgrowth in the small intestine. <sup>14</sup>C-glycine is released from the BA, absorbed into the portal circulation, rapidly metabolized in the liver and the end product is then exhaled into the breath where it is measured as an early peak of <sup>14</sup>CO<sub>2</sub>(12). If the <sup>14</sup>C-BA enters the colon and is not reabsorbed in the terminal ileum, it will be deconjugated by colonic bacteria. A smaller proportion will remain intact to be excreted in stool therefore stool collection after the breath test can identify BAD(49). A disadvantage to this

test is that positive breath exhalation at 2-4 hours will not differentiate between BAD and small bowel bacterial overgrowth. As well as concerns over radiation exposure, there is the labour intensity and complexity of the test, which limits its use in clinical practice(49).

#### *<sup>75</sup>Selenium Homotaurocholic Acid Test (<sup>75</sup>SeHCAT)*

This test was introduced to the UK in 1981 and measures BA pool loss through calculating the retention of radiolabelled (<sup>75</sup>Se) synthetic homocholic acid conjugated with taurine 7 days after ingestion(50). [<sup>75</sup>Se]Tauroselcholic acid is a BA analogue, which demonstrates identical physiological behaviour to natural BA conjugates(51). It is resistant to passive diffusion and bacterial degradation and therefore can enter the enterohepatic circulation through reabsorption via the terminal ileum or pass through the colon unaltered to be excreted in the stool(49). 97-100% of [<sup>75</sup>Se]Tauroselcholic acid is excreted with a biological half-life of 2.6 days and 3% is eliminated with a mean half-time of 62 days(44). Retention rates are expressed as a percentage of the original value of compound retained and are used to grade severity of BAD with cut-offs of 5%, 10% and 15% demonstrating mild, moderate and severe disease respectively. However, these thresholds are not standardized or validated(50).

The patient ingests a capsule of <sup>75</sup>selenium homotaurocholic acid (gamma radiolabeled BA) and after one hour, by which time the <sup>75</sup>selenium has distributed in the gut, a baseline scan is taken and represents 100% retention. A follow up scan is conducted on day 7 where the percentage of remaining <sup>75</sup>selenium homotaurocholic acid in the body is calculated. This is achieved by dividing the amount of radioactivity from <sup>75</sup>selenium on subsequent scans by the baseline scan on day 1(49).

The advantage to this test is that gamma emission, with a short half-life and decreased radiation to extra-abdominal organs is used(49). The main disadvantage is the limited availability of this test and the requirement for multiple visits.

Quantifying severity of disease with the SeHCAT scan has been found to help predict response to BAS with a decreased response noted as severity is reduced(52). In a systematic review of 20 studies considered for the assessment of the value of the SeHCAT test in predicting response from BAS, a positive test in patients with chronic diarrhoea established average response rates of 85%, 73% and 72% for cut-offs at 5%, 10% and 15% respectively. For patients with chronic diarrhoea and a negative SeHCAT test, the response rate was 14% at a cut-off at 5% and 0% at a cut-off of 15%. The test demonstrated greater response rates in those patients with underlying Crohn's disease and a positive SeHCAT test with response rates being 95% at a cut-off of 5% and 86-89% at a cut-off of 15%(44).

A sensitivity of 94% and specificity of 100% have been reported for the use of this test(53). The highest specificity from the SeHCAT test is reported in patients with severe BAD with cut-off levels of <5%-<8%. A higher sensitivity with decreasing specificity is seen in patients with both moderate and severe BAD at the cut-off level of <15%(15).

Despite being the accepted gold standard to diagnose BAD, the SeHCAT scan is underutilized in the UK due to lack of guidance on its role as a diagnostic tool, the prohibitive cost of approximately £210 per patient and the variation amongst clinicians in diagnosing BAD with some performing a diagnostic trial of BAS(54). There is significant heterogeneity amongst UK hospitals in the interpretation on SeHCAT results with lack of consistency in centres utilising differing criteria for defining an abnormal SeHCAT result, impacting on subsequent patient management. Patients with BAD type 1 were found to demonstrate the highest proportion of centre-defined abnormal SeHCAT results(50).

#### *Serum 7 $\alpha$ -hydroxy-4-cholesten-3-one (C4)*

Serum C4 is a simple blood test measuring BA synthesis and is a downstream product of CYP7A1. 90% of BA synthesis is regulated by the enzyme CYP7A1(12). Standardised specimen collection is required due to diurnal variability in C4 with peak concentrations occurring postprandially at

13:00 and 21:00, lasting for 1.5-3 hours(55). Typically, blood is drawn after an overnight fast and C4 is isolated using liquid chromatography-tandem mass spectrometry. An elevated serum C4 concentration has been defined as being >60.7ng/mL(49).

When compared to the SeHCAT test, the C4 assay has a lower performance with sensitivity of 90%, specificity of 79%, negative predictive value of 98% and positive predictive value of 74% in diagnosing BAD(49). The test is limited in its use as it remains unknown whether factors such as shift work, age and emotional states may affect the circadian rhythm and therefore the synthesis of BAs(49). Disadvantages to the test include the possibility of a false positive result in patients with liver disease or those who are being treated with statins(12).

#### *Faecal BAs*

Functional diarrhoea and IBS-D have increased secretory BAs (CA, CDCA, DCA) and the measurement of these have proven to be technically challenging(56). Due to diurnal variation in BA synthesis, multiple stool collections over 3-5 days are required to quantify faecal BAs and diagnose BAD(57, 58). The performance of this test is unknown.

Enzymatic and chromatographic approaches are used to measure faecal BAs. An enzymatic 3 $\alpha$ -steroid dehydrogenase assay quantifies faecal BAs indirectly to oxidise deconjugated BAs and produce reduced nicotinamide adenine dinucleotide (NADH) which is then measured biochemically(12). Chromatographic approaches include the use of GC-MS, liquid chromatography-tandem mass spectrometry, or HPLC-mass spectrometry(49).

#### *Serum FGF-19*

Fasting serum levels of the biomarker FGF-19 have found to correlate with SeHCAT retention and inversely with C4 concentrations(59). FGF-19 is synthesized in the ileum and is involved in the negative feedback of BA

synthesis by acting in the liver to inhibit the crucial BA synthetic enzyme CYP7A1(60). The median FGF-19 serum value has been found to be 65% of the median in the control group of a study that presented with similar symptoms and had normal SeHCAT retention. Lower FGF-19 levels were also found in patients with severe BAD (SeHCAT retention value 0-5%) as well as in the obese patients with primary BAD(11). The microRNA miR-34a is found to be increased in obesity and has demonstrated a role in FGF-19 responses via regulation of  $\beta$ -Klotho expression(61). Significant heterogeneity was evident in FGF-19 levels in patients with BAD and a possible mechanism for this may be defective hepatic responsiveness to FGF-19(59). Therefore, this complex interplay of mechanisms may contribute to the pathogenesis of BAD.

To detect a SeHCAT cut-off of <10%, a fasting FGF-19 level of 145 pg/ml has demonstrated a 61% positive predictive value and a 82% negative predictive value(11).

#### *Urine Volatile Organic Compounds (VOCs)*

This test involves detection of VOCs in urine samples, utilising the technology of an electronic nose in combination with a Field Assymmetric Ion Mobility Spectrometer (FAIMS) as well as a Gas Chromatography Mass Spectrometry (GC-MS). VOC signatures are the resultant products of fermentation by the intestinal microbiome and this is covered in further detail in section 1.3.1. Linear discriminant analysis has been able to separate out the VOC profiles of patients with BAD from both healthy controls and patients with ulcerative colitis with achievement of statistical differences between the groups. In this study, GC-MS suggested the presence of two chemical compounds in patients with BAD, 2-propanol and acetamide, produced by the cleavage of BAs by gut bacteria, may account for the differences found(54). Advantages to the utility of VOCs as biomarkers in the diagnosis of BAD are that they are non-invasive, portable and relatively inexpensive.

### **1.1.6 Management of BAD**

The mainstay of treatment is a low fat diet coupled with anion exchange resins - bile acid sequestrants (BAS). Patients may also utilize conventional anti-diarrhoeals such as Loperamide, which have the advantages of being relatively inexpensive, available in tablet form, have no effect on the bioavailability of co-administered medications and being better tolerated than BAS(27). They are prescribed by a quarter of British Gastroenterologists for the treatment of BAD(40).

In underlying causes of BAD (e.g. Crohn's), therapy should be targeted towards the specific condition, although in many patients, there are no particular aetiologies that are identified (such as in primary BAD) or are treatable (e.g. post cholecystectomy). Despite BAD being a prevalent condition, there is currently a lack of national guidance available on the appropriate management of patients who may benefit from targeted therapeutic intervention. NICE have recognised this and recommend that further research into the efficacy and tolerability of treatment for BAD is required(27).

#### *Low Fat Diet*

The use of low fat diets (<30-40g fat/day) is not well described. Ingestion of dietary fat has demonstrated an increase in faecal BA excretion rate(62). In patients with mild BAD, a low fat diet may be considered with or without the addition of medium-chain triglycerides, as a calorific supplement, which do not require BAs for solubilisation(46).

A study of patients with BAD secondary to pelvic irradiation therapy for malignant gynaecological tumours, demonstrated that a low fat diet of <40g/day for 3-6 months resulted in a decrease in a faecal extraction of BAs with relief of symptoms(63). A more recent, larger study agreed with these results. Dietary intervention of providing 20% of energy from fat was undertaken, reducing the daily mean fat intake by a third. This led to a significant improvement in clinically important symptoms including bowel

frequency, urgency, lack of control, flatulence, abdominal pain, steatorrhea, borborygmi and bloating(64). Formal dietetic assessment is required and compliance may be low due to poor taste(27).

#### *Bile Acid Sequestrants (BAS)*

BAS are positively charged, non-digestible resins, with high affinity in binding to BAs in the intestine to form insoluble complexes, which are then excreted in the faeces. Before the advent of statins, these medications were used in patients with raised cholesterol, as by reducing the enterohepatic circulation of BAs, cholesterol is converted to BAs in the liver. This results in a reduced hepatic cholesterol content, which in turn, increases expression of the LDL-receptor, thereby lowering the serum LDL-cholesterol concentration(65).

There are currently three commercially available BAS: Colestyramine, Colestipol and more recently, Colesevelam. Colesevelam is unlicensed for the use in BAD and coupled with the fact it is more expensive compared to the other BAS, it is rarely used in clinical practice. Both Colestyramine and Colestipol have been used in the management of cholestatic pruritus, particularly in Primary Sclerosing Cholangitis(66). The emerging role of BAS in patients with type 2 diabetes mellitus, metabolic syndrome and insulin resistance is also being recognised(67, 68).

BAS are also capable of binding other compounds therefore can impair absorption of fat-soluble vitamins (A,D, E and K) and medications including diuretics, digoxin, warfarin and beta-blockers(69). This effect may be overcome by administering BAS either 1 hour after or 4-6 hours before other drugs and monitoring levels of fat-soluble vitamins periodically. Colesevelem has the potential benefit of not affecting the bioavailability of other drugs and vitamins<sup>71</sup>. This is due to its individual structure (compared with the other BAS) of a polyallylamine crosslinked with epichlorohydrin and alkylated groups, causing an 'open' structure with side chains that maximize binding with BAs and reduce interactions with other medications(65).

Despite having a high safety profile and being cheap, BAS are limited in their use through poor compliance due to the gastrointestinal side effects they may incur in patients. These include abdominal pain, constipation, bloating, nausea and flatulence, which may lead to discontinuation of treatment in 40-70% of patients(70). They also have poor palatability given they only exist in powder/granule forms, which need to be dissolved into a paste. Colesevelam is better tolerated due to its more gelatinous consistency(71).

A recent systematic review of the management of BAD, the largest to date, found Colestyramine treatment to be effective in 70% of patients (range 63%-100%) who maintained good clinical response at follow up. An association between response and severity of BAD was not evident(27). In a randomized controlled trial, Colesevelam was investigated as first line therapy which found that it was associated with firmer stool consistency, a moderate increase in the 24-hour colonic transit time and 'greater ease of stool passage'(72). The use of Colesevelam as first- and second-line therapy (after failure of Colestyramine) has been investigated with 87% of patients declaring success after reporting an improvement in symptoms and continuing long-term treatment. As second-line therapy, Colesevelam was deemed successful in 57% of patients who maintained good clinical response at follow-up(73). Treatment with Colesevelam has been significantly associated with the severity of BAD, based on serum 7 $\alpha$ C4 levels(72).

### *FXR Agonists*

A proof-of-concept study investigating the utility of obeticholic acid in patients with BAD was recently undertaken(74). Obeticholic acid is an FXR agonist that is 100 times more potent than CDCA, a natural FXR agonist(75). In patients with primary BAD, where reduced levels of FGF-19 have been demonstrated, the use of an FXR agonist was hypothesized to stimulate FGF-19 production in the ileum. CDCA has been shown to induce FGF-19 transcript expression in human ileum explants(76). In patients with



primary BAD, the study exhibited an increase in the baseline fasting median FGF-19 level after treatment with obeticholic acid with levels being similar to those found in healthy controls. 90% of patients had a median increase of 71% over baseline FGF-19 levels. Fasting C4 and BA values were reduced after treatment with obeticholic acid in patients with primary BAD. Stool frequency and consistency improved with treatment in patients with primary BAD and those with secondary BAD resulting from shorter ileal resections (<45cms). An improvement was noted in the symptoms of urgency in patients with both primary and secondary BAD and pain in patients with secondary BAD(74).

## **1.2 Diarrhoea predominant - Irritable Bowel Syndrome (IBS-D)**

### **1.2.1 Irritable Bowel Syndrome (IBS) and Burden of Disease**

IBS is a multifactorial, chronic functional bowel disorder that is the most commonly diagnosed gastrointestinal condition. There is an absence of a physiological or a demonstrable structural abnormality to explain the symptoms of IBS sufferers(77). The diagnosis of IBS is usually confirmed once organic (pathological) disease has been excluded and after a negative diagnostic evaluation, a patient being diagnosed with IBS has a less than 5% risk of receiving an alternative organic diagnosis in the future(78). IBS accounts for a significant proportion of referrals to secondary care. Referrals for IBS in Northern England were responsible for 36% of all new patient appointments with each patient requiring an average of two visits(79). Despite IBS having the ability to be diagnosed and managed within primary care, 30% of patients in the UK are referred to secondary care; two thirds to a physician and a third to the surgeons(80). This may either be to exclude other potential diagnoses or for further management. A UK study demonstrated that three months after seeing a Gastroenterologist for IBS, patients only experienced a small and not statistically significant improvement in quality of life, which was not maintained(81).

The condition can be debilitating in some patients whereas others may only experience mild or moderate symptoms(82). It is a disease based on symptoms, characterised by the presence of abdominal pain or discomfort accompanied by altered stool form or frequency(83). The subtypes of IBS are categorized by the Rome IV criteria (discussed in section 1.2.4), according to the predominant symptom and include: IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U)(84). Somatic co-morbidities are often associated with IBS, including somatic pain disorders (chronic fatigue syndrome, fibromyalgia and chronic pelvic pain) psychiatric conditions (anxiety, major depression) and other function gastrointestinal disorders (dyspepsia, gastroesophageal

reflux disease)(85, 86). Several of these co-existing functional conditions occur in approximately half of all patients with IBS and exist almost twice as often as in the general population(87-89). IBS patients with these somatic disorders report symptoms that are more severe than those in patients with IBS alone(88, 90).

IBS is a chronic, relapsing condition, representing a considerable burden to individuals, society and healthcare systems. Patients utilize more healthcare resources than those without IBS beyond the first year following diagnosis for gastrointestinal symptoms and non-gastroenterology related care(91). IBS generates a significant economic burden secondary to the costs of consultations, investigations, prescribed and over-the-counter medications, and sickness absence from work(92-94). In the United States, the total direct and indirect expenditures exceed \$20 billion with an estimated direct cost per patient being between \$742 and \$7547(91, 95). 12% of patients stop work due to their IBS(96). Reductions in other dimensions affecting quality of life in patients with IBS may also occur with one study reporting 68% of sufferers missing out on an activity or social event each week(97). The severity of bowel and psychological symptoms produces the most significant effect on reducing quality of life in patients with IBS(98).

The natural history and symptoms of IBS vary over time and patients may migrate between different IBS subtypes(99). Usually, transition is from IBS-C or IBS-D to IBS-M whereas switching between IBS-C and IBS-D occurs less commonly(100). Although IBS assumes no attributable mortality, it still bears significantly on quality of life(101, 102). A systematic review demonstrated that during long-term follow-up of clinic-based IBS patients, 30%-50% remained unchanged, 12%-38% improved and 2%-18% worsened. Worse outcomes were predicted by various factors including longer duration of disease, higher somatic scores, previous surgery and co-existing anxiety and depression(78).

The global prevalence of IBS is 11%(77). The lowest prevalence of IBS is found in Southeast Asia (7%) with the highest occurring in South America

(21%)(77). In Western populations, the prevalence of IBS is up to 20% with an incidence of 1-2% in community samples(77, 103). Data from the United States report that 30% of patients with symptoms will consult, with 80% of these patients having IBS-D(104). In a meta-analysis examining the prevalence of IBS according to each of the 4 subtypes, even distribution was demonstrated. 22% of patients had IBS-C, 23% had IBS-D, 24% had IBS-M and 22% had IBS-U. The prevalence of IBS was also found to be higher in females (14%) compared to males (8.9%). With regards to socioeconomic status, there was no significant difference in the prevalence of IBS found in patients of higher socioeconomic status compared to those of medium or lower socioeconomic statuses(77).

### **1.2.2 Risk Factors for IBS**

The greatest documented risk factor for IBS is female gender with an odds ratio of 1.67. Women were more likely to exhibit IBS-C and less likely to meet criteria for IBS-D than men(105). Reasons underlying the excess of IBS seen in women may include differences in gender health seeking behaviour, gender differences in access to health care and biological functions (e.g. hormonal regulation of gut function)(82). Women with IBS are more likely to have suffered sexual, verbal or physical abuse, which may contribute to the development of IBS through dysfunction of the brain-gut axis and mucosal immune system(106).

Clustering of IBS within families has been reported and is driven by social learning, shared household environmental exposure and genetics(107, 108). Individuals with a biological relative with IBS have a twice as high relative risk of developing IBS(109). Twin studies have demonstrated that having a mother or father with IBS is a stronger predictor for an individual having IBS than having a twin with the condition(110).

IBS occurs in patients across all age groups, with no difference observed in the frequency of subtypes by age(111). With advancing age (>50 years), the incidence of IBS decreases but is similar in children and adolescents compared with adults and does not necessarily transition from childhood to

adult life(77, 112). Recurrent abdominal pain in childhood may reflect learned illness behaviours in some IBS patients, initiating persistent changes in the brain-gut axis which leads to the unconscious perception of interoceptive input from the gastrointestinal tract(113, 114). Patients above the age of 50 years report milder pain however their quality of life is deemed worse(111). Patients above the age of 65 years are likely to have symptoms for at least one year before they consult whereas younger patients under the age of 65 years report a significantly shorter history of symptoms(115).

An association between preceding gastrointestinal infection and IBS has been demonstrated with a reported prevalence of 10%(116). Female sex, younger age, premorbid psychological conditions and the severity of the initial infection are risk factors for developing post-infectious IBS(82). Unlike typical IBS, post-infectious IBS spontaneously resolves in approximately half of patients within 6-8 years of the index infection(117).

Personal, psychological, social and somatic factors have all found to be associated with an increased risk of patients developing IBS and are outlined in table 1. Less well recognized factors are marked (\*) and are based on single studies(82).

**Table 1: Factors associated with risk of developing IBS**

<b>PERSONAL</b>			
Female sex	Age >50 years	Birth cohort*	Breast feeding (<6 months)*
Herbivore pet in childhood*	Low birth weight*	Low body mass index*	
<b>PSYCHOLOGICAL</b>			
Illness behaviour	Low quality of life	Acute psychological stress	Stressful life events
Sexual/physical abuse history	Anxiety/depression/somatisation	Intimate partner violence*	Addictive behaviour*
<b>SOMATIC</b>			
Gastrointestinal infection	Somatic symptoms (e.g. joint pain, migraine)	Endometriosis	Abdominal obesity
Left-sided diverticular disease	Antibiotic use	Abdominal surgery	Spicy food consumption*
Sleep problems*	Low exercise level*		
<b>SOCIAL</b>			
Childhood socioeconomic status	Family history of substance abuse	Family history of mental illness	Insufficient autonomy at the workplace*
Shift work*	Marital status (never married)*	Increasing number of family members*	Childhood war exposure*

### 1.2.3 Pathophysiology of IBS-D

The pathophysiology of IBS-D is heterogenous and some of the key factors contributing to the aetiology are outlined below:

#### *Diet*

The relationship between diet and IBS is ill defined and diet may trigger IBS symptoms and/or induce alterations in gut microbiota, permeability and pro-inflammatory responses(85, 118). Osmotically active, short-chain carbohydrates (including lactose, fructose, sugar alcohols, fructans and galactans) may result in increased fermentation in the bowel, exacerbating symptoms in patients with IBS who have underlying abnormalities in gut sensation and function(119). Postprandial exacerbations of symptoms either as an immediate or deferred reaction have been reported in 50% of patients with IBS(120). Many patients associate one or more foods with the onset of symptoms and the most implicated food items include wheat, milk, caffeine, fructose, alcohol, certain meats, spicy and fatty foods, dairy products and grains(121). A high fat intake in IBS patients is associated with a heightened colonic motor response to eating and increased visceral sensitivity(122). A relationship between IgG antibodies and IBS symptoms has been reported in patients with IBS-D where exclusion of specific IgG-associated foods resulted in a significant decline in the symptoms of abdominal distension, pain and bloating(123).

An exaggerated postprandial serotonin response may account for the symptoms of urgency and diarrhoea seen in IBS-D as serotonin stimulates receptors responsible for intestinal secretion and peristalsis(121, 124). This occurs as elevated levels of postprandial serotonin have been demonstrated in patients with IBS with decreased serotonin reuptake resulting from diminished affinity for the reuptake transporter protein(124, 125). Accelerated colonic transit where an increased number of brief (<15 seconds) colonic contractions, alterations in small intestinal motor function (e.g. increased frequency of duodenal and jejunal contractions, migrating motor complexes and ileal distension) have been reported in patients with

IBS-D, however, whether this is secondary to a disruption in serotonin signaling or other mechanisms remains unclear(126-128).

The influence of dietary factors on the immunological phenomena of the gut microbiota in IBS is observed by the impact of probiotic administration and will be discussed in section 1.3.4. The overproduction of hydrogen by intestinal microbes has been associated with IBS symptoms, especially flatulence and abdominal pain(129). Methanogenic Archae, which efficiently remove hydrogen gas in the intestine, have demonstrated decreased levels in patients with IBS-D(130). A low FODMAPs (fermentable, oligo-, di-, monosaccharides and polyols) diet, which may be used to improve symptoms in patients with IBS-D, has been found to reduce absolute counts and total abundance of intestinal bacteria but not relative abundance of specific bacterial groups. Lower relative abundances of butyrate-producing bacteria and *A. muciniphila*, and a significantly higher abundance of *R. torque* have been reported in the low-FODMAP diet in comparison to a high-FODMAP diet(131).

Apart from investigating the impact of the FODMAPs diet on the intestinal microbiota, there is a lack of data examining the functionality effect of this diet and other dietary interventions on the gut microbiome in patients with IBS(122). Animal studies have illustrated alterations in diet have a considerable influence on intestinal microbial composition, function, and effect. Mice fed a high fat diet were found to have impaired intestinal mucosal barrier integrity secondary to modification of the BA profile with an increase in the concentration of DCA and decrease in the proportion of a potentially cytoprotective tertiary BA, ursodeoxycholic acid (UDCA). The decrease in UDCA was associated with disruption of the intestinal barrier most likely due to the increased ability of cytotoxic BAs like DCA to induce barrier dysfunction. DCA has been recognised to disrupt lipid bilayers while the hydrophilic BA UDCA stabilizes them and protects mitochondria against DCA-induced reactive oxygen species production(132). Another mouse study found that consumption of a high saturated fat diet stimulated the expansion of the sulphite-reducing microorganism, *Bilophila wadsworthia*.



This was related to a proinflammatory T helper type 1 immune response with an increased incidence of colitis in genetically susceptible mice that lacked IL-10(133).

### *Epithelial Barrier*

Disruption of the intestinal epithelial barrier permits increased antigenic invasion resulting in the activation of the mucosal immune systems, which may progress to IBS-D(134, 135). The inflammatory infiltrate is composed of increasing numbers of activated mast cells and mast cell products (mainly neurotransmitters and inflammatory mediators), which have been located in the terminal ileum, and the proximal and distal colon of IBS patients(136-139). Mast cells are involved in the regulation of visceral sensitivity, intestinal motility and epithelial and mucosal gut barrier function(140). Acute and chronic stress promotes mast cell activation and the presence of chronic stress in patients with IBS-D has been found to significantly contribute to symptom intensity and duration of these symptoms(141-143). In IBS-D patients, activation of mast cells, measured by expression of tryptase mRNA, exhibited correlation with stool frequency and consistency. Poor correlation was seen with absolute numbers of mast cells, implying that mast cell activation rather than numbers influence bowel habit(144).

Tryptase, one of the mediators released by mast cells, can cause morphological changes in the epithelial gut barrier by activating protease-activated receptor 2 on epithelial cells, resulting in modulation of tight junction (TJ) proteins and increases in permeability through paracellular pathways(145). The jejunal mucosa of IBS-D patients has demonstrated structural abnormalities at the intercellular apical junction complex (AJC) with perijunctional cytoskeletal condensation and enlarged apical intercellular distance. This was associated with molecular and gene alterations, which identify mechanisms regulating AJC function. An increase in the expression of the CLDN2 protein and decreased expression of CLDN3 and CLDN4 has been observed(146). CLDNs (claudins) and OCLN (occludins) are TJ transmembrane proteins, which form the structural core of the TJ and are associated with the perijunctional cytoskeleton

(predominantly actin and myosin), which is vital for the regulation of paracellular permeability(135). CLDN2 has been associated with cation-selective channel activity and increased intestinal permeability, whereas CLDNs 3 and 4 reduce permeability through the paracellular space, strengthening the epithelial wall barrier(147, 148). The net modification observed in the expression of the components of the CLDN protein family would result in an increase in the permeability of the intestinal wall barrier(146).

The phosphorylation status of OCLN is related to its distribution in epithelial cells(149). OCLN has been located in the cytoplasm of jejunal enterocytes, suggesting redistribution of the protein with internalization seen whereas in healthy subjects, it was predominantly observed in the TJ(146). Internalisation and phosphorylation of OCLN has demonstrated a disruption in the integrity of the AJC, resulting in increased intestinal permeability(150). Increased myosin light chain (MLC) phosphorylation has been observed in IBS-D patients, which has been linked to AJC disassembly and disruption of the epithelial wall barrier(146, 151, 152).

Lastly, the presence of HLA-DQ2 or DQ8 genotype appears to render IBS-D patients with increased susceptibility to gluten altering mRNA expression of TJ proteins and increased small bowel permeability(153).

### *Bile Acids*

BAs are regarded as endogenous laxatives that stimulate colonic motility, increase mucosal permeability, reduce net fluid and electrolyte absorption and encourage net secretion across colonic epithelial cells(37, 154, 155).

Increased colonic exposure to BAs in patients with predominantly IBS-D and IBS-M, influences colonic transit time and bowel habit. A subset of patients with IBS who had an abnormal SeHCAT retention value and/or increased C4 levels, indicating increased BA exposure to the colon, were observed to have increased bowel frequency, accelerated colonic transit time and rectal hyposensitivity(156). This is supported by another study demonstrating increased faecal primary BAs in patients with IBS-D compared to healthy

controls. This correlated with increased stool frequency and reduced stool consistency(157).

### *Microbiome*

The role of the gut microbiome in IBS is discussed in section 1.3.4.

### *Brain-Gut-Immune Axis*

The gut, brain, intestinal microbiota and immune systems demonstrate reciprocal associations in health and disease(82). A combination of hormonal, neuronal and immunological pathways allows the brain to modulate the sensory, motor, secretory and autonomic functions of the gastrointestinal (GI) tract and this communication of the brain with the GI tract is bidirectional(158). Through the autonomic nervous system and hypothalamus-pituitary-axis (HPA), the brain can influence various mechanisms, which have been reported to be dysregulated in IBS. These include intestinal epithelial permeability, immune function, intestinal motility and fluid secretion and composition of the intestinal microbiome(159-161).

The enteric nervous system (ENS) and central nervous system (CNS) influence many gastrointestinal functions via communication along the vagal and autonomic pathways(158). Increased parasympathetic tone and adrenergic dysfunction have been demonstrated in patients with IBS-D when compared to those with IBS-C, which may influence colonic motor activity and IBS subtype(162).

Functional signaling between immune cells and nerves predominantly occurs in between the epithelial and submucosal layers of the intestinal wall where there is a high concentration of immune cells – mainly, mast cells, T lymphocytes and macrophages. Cytokines and mediators released by these immune cells interact with extrinsic nerves, enteric nerves and glial cells(82). Mucosal mediators from patients with IBS evoke increased activation of somatic pain and visceral pathways when applied to intestinal preparations isolated from rodents compared with healthy controls(163, 164). Mucosal mediators derived from patients with IBS and visceral

hypersensitivity activated spinal nociceptors acutely when given to animal models but this observation did not occur in normosensitive patients with IBS(165). Furthermore, chronic exposure to soluble mediators from colonic biopsies from patients with IBS-D has demonstrated sensitization of nociceptive neurons(166).

The hypothalamic-pituitary-adrenal (HPA) axis can be activated in response to environmental stress by the key activating hormone, corticotrophin-releasing factor (CRF), secreted from endocrine cells in the paraventricular nucleus of the hypothalamus(158, 167). Animal studies have demonstrated stress induced release of CRF stimulates colonic secretion, intestinal motility and visceral sensitivity(167). CRF stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH), which in turn, induces secretion of cortisol from the adrenal cortex. Cortisol has an anti-inflammatory role on the GI immune and systemic system. Reduced cortisol levels and response and heightened vagal activation have been observed in patients with IBS-D(168).

#### **1.2.4 Diagnosis and Symptoms of IBS**

Variations in form and frequency of bowel habits, along with having 3 or more different stool forms per week have been shown to discriminate IBS-D from other organic gastrointestinal disease(169). Other symptoms, although non-specific, support the diagnosis of IBS and include abnormal stool frequency (>3 per day or <3 per week), abnormal stool passage (urgency, feeling of incomplete evacuation, straining), abnormal stool form (loose/watery stool or hard/lumpy), bloating/abdominal distension and passage of mucus(170). A post-prandial exacerbation of symptoms is common in IBS but not exclusive to the diagnosis(171).

Symptoms of IBS-D may mimic other organic gastrointestinal diseases and therefore it is important to screen for them. Patients with IBS symptoms have a 4-fold increased likelihood of biopsy proven coeliac disease and therefore routine screening in these patients particularly those with IBS-D,

should be undertaken with IgA TtG +/- quantitative IgA(172, 173). A small subset of patients with suspected IBS-D may have microscopic colitis therefore when colonoscopy is performed, random colonic biopsies should be carried out(173). As mentioned earlier, a third of patients with IBS-D symptoms have BAD therefore testing for this condition with a SeHCAT scan or any other diagnostic tool that is available is advised. Lastly, it is important to consider inflammatory bowel disease (IBD) in the differential diagnosis of patients with IBS-D symptoms. Studies suggest that the Rome criteria for IBS may be fulfilled in more than a third of patients with IBD(174). Therefore, the use of faecal calprotectin, which has been found to be cost-effective in identifying IBD, is advisable(175). A recent meta-analysis demonstrated that a CRP of  $\leq 0.5$  and faecal calprotectin of  $\leq 40$ , excludes IBD in patients with symptoms of IBS and that the addition of these two biomarkers, along with symptom-based criteria, may further support the diagnosis of IBS(176).

A recent systematic review of the accuracy in diagnosing IBS with diagnostic criteria, biomarkers and/or psychological markers demonstrated that performing a combination of these tools was a more effective way in diagnosing IBS compared with using diagnostic criteria alone. The combination in achieving the best positive likelihood ratio was faecal calprotectin, intestinal permeability and Rome I criteria(177).

### *Diagnostic Criteria*

The diagnosis of IBS relies on the exclusion of organic pathology and the presence of symptoms in alliance with diagnostic criteria, the latest version being the Rome IV criteria, which is the current international gold standard. These criteria include abdominal pain or discomfort associated with altered bowel habit. Stool consistency may be assessed with the Bristol Stool Form Scale, which is a validated tool in reporting of stool appearance from a score of 1 (hard and lumpy stool) to 7 (entirely liquid)(178). There is currently no valid biomarker for IBS and although patients often undergo a

series of investigations to exclude organic disease, the current recommendation is to base diagnosis on symptoms(82).

The first attempt at diagnostic criteria for IBS was the Manning criteria, published in 1978(179). The experience gained from the Manning criteria aided the development of the Rome Foundation criteria, which have been found to be 69-96% sensitive and 72-85% specific for the diagnosis of IBS(180). All these criteria require symptoms to be chronic and recurring and occur for  $\geq 3$  days per month (now  $\geq 1$  day per week in the new Rome IV criteria) in the past  $\geq 3$  months with symptom onset  $\geq 6$  months before the diagnosis (Rome III and IV criteria)(82). The Rome III criteria have only been validated in one study(181). In addition, the Rome III criteria have been criticized for separating out those with IBS-D from patients with functional diarrhoea with overlap of the two conditions occurring in one in four patients. Those with IBS-D were found to have differing characteristics to those with functional diarrhoea: IBS-D patients were of a significantly younger age, more were female, of a lower BMI, more often reported bloating, had a higher rate of education to university or post-graduate level and demonstrated higher levels of anxiety and somatization. Frequency of diarrhoeal symptoms was no different between the two groups of patients(182).

Since publication of the Rome III criteria in 2006, there have been several changes to the Rome IV diagnostic criteria for IBS, which are as follows(183):

1. Removal of the term 'discomfort' from the criteria and use of only the term 'pain'.
2. Increased minimum frequency threshold of abdominal pain from 3 days/month in Rome III to 1 day/week in Rome IV.

3. Relief of abdominal pain/discomfort after defecation was previously required in Rome III, which is no longer required in Rome IV and has now been replaced to pain related to defecation.

4. Stool consistency and change no longer require starting at the 'onset' of abdominal pain/discomfort. Demonstration of an association between the symptoms in Rome IV is now required.

5. A change in identifying the IBS subtypes where previously, Rome III criteria required the proportion of total stools using the Bristol Stool Chart Scale to classify IBS. As patients may have greater duration of normal stool frequency, a majority was being classified as IBS-U compared to the other subtypes. Rome IV now relate the diagnostic criteria to the proportion of days with symptomatic stools (i.e. loose/watery) rather than all stools (including normal ones). This has resulted in the IBS-U group being reduced significantly.

The Rome III and IV criteria are demonstrated in table 2.

**Table 2: Rome III and IV criteria in diagnosing IBS**

Rome Criteria	Rome III (2006)(83)	Rome IV (2016)
<b>Symptoms</b>	<p>Recurrent abdominal pain or discomfort at least 3 days/month in the last 3 months, associated with <math>\geq 2</math> of the following:</p> <ol style="list-style-type: none"> <li>1. Improvement with defecation</li> <li>2. Onset associated with change in stool frequency</li> <li>3. Onset associated with change in stool form (appearance)</li> </ol>	<p>Recurrent abdominal pain, on average, at least 1 day per week in the last 3 months, associated with <math>\geq 2</math> of the following:</p> <ol style="list-style-type: none"> <li>1. Related to defecation</li> <li>2. Associated with change in stool frequency</li> <li>3. Associated with change in stool form (appearance)</li> </ol>
<b>Duration of Symptoms</b>	Criteria should be fulfilled for at least 3 months with symptom onset $\geq 6$ months prior to diagnosis.	

### ***Biomarkers***

As discussed earlier, diagnostic criteria alone perform only modestly in predicting the diagnosis of IBS and therefore, further research is being conducted into developing novel biomarkers, which may aid in the diagnostic work-up of IBS.

#### ***Serum biomarkers***

In 2009, ten serum biomarkers were incorporated into a diagnostic algorithm and assessed in differentiating IBS from non-IBS. These include Interleukin- $1\beta$  (IL- $1\beta$ ), Growth-related oncogene- $\alpha$  (GRO- $\alpha$ ), Brain-derived neurotrophic factor (BDNF), Anti-*Saccharomyces cerevisiae* antibody (ASCA IgA), Antibody against CBir1 (Anti-CBir1), Antihuman tissue transglutaminase (tTG), Tumour necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), Antineutrophil cytoplasmic antibody (ANCA), Tissue inhibitor of metalloproteinase-1 (TIMP-1) and Neutrophil gelatinase-associated lipocalin (NGAL). The sensitivity and specificity of this algorithm was 50% and 88%



respectively(184).

More recently, 34 gene expression and serology markers (including the 10 biomarkers utilized in 2009) were combined with 4 psychological measures to differentiate IBS from health and between subtypes of IBS. The other biomarkers included in this study were histamine, prostaglandin E2 (PGE2), serotonin, tryptase, substance P, interleukins 6, 8, 10 and 12 as well as a host of gene expression markers. This panel had a sensitivity and specificity of 81% and 64%, respectively in differentiating between IBS and health. A subset of four biomarkers (anti- tTG, histamine, ZNF326 and RNF26) contributed to the majority of the performance. With regards to IBS subtypes, the best differentiation was observed between IBS-D and IBS-C (AUC = 0.92). The addition of the psychological markers did not improve discrimination further between the IBS subtypes but did yield an incremental discrimination in IBS from health(185). None of these biomarkers mentioned are currently used in clinical practice.

### ***Volatile Organic Compounds (VOCs)***

This is discussed in section 1.5.3.

### ***Granins***

Secretogranins (Sg) and chromatogranins (Cg) are proteins located in secretory cells of the endocrine, enteric and immune systems, which are thought to reflect their activity. Faecal levels of SgII, SgIII, CgA and CgB have been investigated in patients with IBS and healthy controls. Higher faecal levels of SgII, SgIII and CgA with lower levels of CgB were seen in IBS patients and this reached statistical significance. SgII demonstrated the greatest discriminatory performance in positively identifying IBS patients(186). Granins are not specific to IBS as they can be observed in other gastrointestinal conditions such as coeliac disease and lymphocytic colitis(187).

### ***Epithelial Gap Density***

Disrupted intestinal permeability may compromise intestinal barrier function,

which is maintained by epithelial cells with tight junctions between the cells(188). An increased epithelial gap density in the terminal ileum in IBS patients has been demonstrated using confocal laser endomicroscopy. A trend towards higher gap density in female and younger patients was observed. A cut-off value of 30 gaps per 1,000 cells was used to define abnormal cell gap density and revealed a sensitivity and specificity of 62% and 89%, respectively(189). This is not exclusive to IBS as patients with IBD have also demonstrated an increased epithelial gap density(190). Furthermore, the diagnostic accuracy of this test is likely to depend on the operator's skill set and experience and the test may only be limited to particular endoscopy units.

### ***Colonic Mucosal Markers***

Immune activation may contribute towards the pathogenesis of IBS. Using quantitative immunohistochemistry on mucosal biopsies, IBS patients have shown a significant 72% increase in colonic immunocytes compared to controls, with increased numbers of CD3+, CD4+ and CD8+ T cells and mast cells. 50% of IBS patients have demonstrated increased immune cells, however this test is limited as a significant immune infiltrate has also been observed in other gastrointestinal diseases, including microscopic colitis and ulcerative colitis(191).

### **1.2.5 Pharmacological Management of IBS-D**

Management of IBS involves a holistic, integrated approach based on reassurance, education, dietary alterations, pharmacotherapy and behavioural and psychological treatment, given the fact that 50-70% of IBS patients report somatic and psychological symptoms(192, 193). In 2009, the American College of Gastroenterology IBS Task force announced that the three classes of medications with the strongest evidence (grade 1B – moderate to high evidence) for efficacy in IBS-D were tricyclic antidepressants (TCAs), antibiotics (i.e. Rifaximin) and the 5HT<sub>3</sub> antagonist Alosetron(194, 195).

#### *a) Anti-motility Agents*

Loperamide, a  $\mu$ -opioid receptor agonist, is frequently used as a first-line agent in IBS-D. Its main benefit is to improve stool consistency, reduce stool frequency and defecation urgency but has less impact on the symptoms of abdominal pain and bloating(194, 196). It works by reducing peristalsis, increasing intestinal transit time and reducing loss of fluid and electrolytes(118). Central adverse effects are avoided as Loperamide does not cross the blood-brain barrier(82). It has shown to be well tolerated, with the only adverse effects reported including constipation, swollen fingers, trouble with sleeping and oral blisters(197-199). Despite Loperamide being an effective antidiarrhoeal drug, there is no evidence to support its use in relieving global IBS symptoms(194).

Eluxadoline, a mixed  $\mu$ -opioid receptor agonist and  $\delta$ -opioid receptor antagonist, reduces contractility and secretion in the gastrointestinal tract and was approved for use in IBS-D patients in the USA in 2015(200). In two phase 3 trials, 23-33% of patients on 75mg and 100mg of Eluxadoline, compared to 16-20% of the placebo group, demonstrated a decrease in abdominal pain and an improvement in stool consistency on the same day for at least 50% of the days for 26 weeks. The most common adverse events observed were nausea, constipation and abdominal pain. The safety concerns of the drug were secondary to the excess rates of pancreatitis(201).

#### *b) Serotonergic Agents (5-HT<sub>3</sub> receptor antagonists)*

These include Alosetron, Ramosetron and Ondansetron and their mechanism of action is considered to occur through inhibition of the ascending excitatory component of the peristaltic reflex and of the high amplitude propagating contractions within the gastrointestinal tract(202). 5-HT<sub>3</sub> receptors have shown an important role in visceral pain, and 5HT<sub>3</sub> antagonists reduce painful sensations from the intestine and slow intestinal transit(203, 204). Randomised, placebo-controlled trials of Alosetron have demonstrated an improvement in stool consistency, stool frequency and

overall IBS symptoms in females, however the effects were limited by a rapid reduction with treatment cessation(205-210). In males, a study observed treatment with Alosetron resulted in improved stool consistency, relief of abdominal pain and discomfort but a lack of effect on stool frequency, bloating, urgency and incomplete evacuation(211). The concerns surrounding Alosetron are based on reports of ischaemic colitis, occurring in approximately 0.1% of patients(212). Ramosetron, is of benefit in both men and women with IBS-D, demonstrating 'a global assessment of relief of IBS symptoms' and been found to be safe(213, 214). Out of the 5-HT<sub>3</sub> antagonists, Alosetron is currently the only medication, which has been recommended for use in females with IBS-D(194).

### *c) Antidepressants*

Selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) improve global IBS symptoms and reduce abdominal pain in IBS patients (NNT = 4)(194). In low doses, TCAs are effective in relieving pain of visceral origin, increasing pain thresholds, relieving concomitant depression and altering intestinal transit times(47, 194, 215). As TCAs prolong intestinal transit time, they are preferentially used over SSRIs for patients with IBS-D(195).

Table 3 demonstrates three controlled trials investigating TCAs and SSRIs in patients with IBS-D(216-218).

**Table 3: Trials investigating TCAs and SSRIs in IBS-D**

Study	Treatment	Study Duration	Population	Results
Vahedi et al, 2008(216)	Amitriptylline 10mg	2 months	All patients with IBS-D (based on Rome II criteria)	Significant reduction in stool frequency and feeling of incomplete evacuation. Improved complete response (loss of all symptoms).
Greenbaum et al, 1987(217)	Desipramine 50-150mg	Crossover study with Atropine and placebo (3 x 6-week test periods)	28 IBS patients (19 with IBS-D, 9 with IBS-C)	IBS-D patients: Decreased stool frequency, diarrhoea, abdominal pain, depression and slow rectosigmoid contractions. 13/19 IBS-D patients improved 'globally'.
Talley NJ et al, 2008(218)	Imipramine 50mg and Citalopram 40mg (double-blind, placebo-controlled, parallel group)	12 weeks	51 IBS patients (37 with IBS-D) – based on Rome II criteria	Imipramine: improvements in bowel symptom severity rating for interference, distress and depression score.  Citalopram not superior to placebo.

(Adapted from Olden KW. Targeted therapies for diarrhoea-predominant irritable bowel syndrome. *Clin Exp Gastroenterol*, 2012)

With TCAs, adverse effects include constipation, dry mouth, dizziness and drowsiness(118). Adverse effects of SSRIs include diarrhoea, nausea and vomiting. As these side effects are common, they may limit patient tolerability(194).

#### *d) Dietary Modification*

Patients with IBS readily specify particular food products that precipitate symptoms however only 11-27% of those are accurately identified when confirmed in formal, blinded food challenge studies(219). Wheat, fruit and vegetables appear to be particularly offending food products and the low FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides

and polyols) diet aims to address this(82). FODMAPs are osmotically active and are poorly absorbed by the small intestine, resulting in the net secretion of fluid into the small intestine, causing distension and abdominal symptoms with increased water delivery to the colon. FODMAPs are also rapidly fermented by the intestinal microbiota, resulting in gas production which gives rise to colonic distension, pain and bloating(220). Two randomized controlled trials have demonstrated an improvement in IBS symptoms with a low FODMAPS diet. One trial specifically showed an improvement in the IBS symptoms of bloating, pain and passage of wind but greater satisfaction was observed in IBS-D patients in particular, where altered faecal frequency and consistency occurred(221).

A very low carbohydrate diet (20g carbohydrate/day) has been observed to improve abdominal pain, quality of life, stool frequency and consistency in IBS-D sufferers(222). A gluten free diet has also been shown to reduce stool frequency in IBS-D patients, the effects being more pronounced in patients with HLA-DQ2 or DQ8 genotype(153).

#### *e) Probiotics*

Probiotics have been defined as 'live microorganisms which when administered in adequate amounts, confer a health benefit on the host(223). There are three randomized controlled trials (RCTs), which have investigated the effects of probiotics in patients with IBS-D and are outlined in table 4(224).

**Table 4: RCTs of probiotics in IBS-D**

Study	Probiotic strains (brand name), dose/duration	Population	Results	P value
Kim HJ et al, 2003(225)	<i>B. longum</i> , <i>B. longum</i> subsp. <i>infantis</i> , <i>B. breve</i> , <i>L. acidophilus</i> , <i>L. paracasei</i> subsp. <i>paracasei</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. plantarum</i> , <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i> (VSL#3)  1 sachet (225 billion lyophilised bacteria) BD for 8 weeks	IBS-D (Rome II)	Reduced abdominal bloating.  No changes in colonic transit	0.046
Ki Cha B et al, 2012(226)	<i>Lactobacillus acidophilus</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus rhamnosus</i> , <i>Bifidobacterium breve</i> , <i>Bifidobacterium lactis</i> , <i>Bifidobacterium longum</i> , <i>Streptococcus thermophiles</i>  1 capsule BD ( $1 \times 10^{10}$ cells/day) for 8 weeks	IBS-D (Rome III)	Increased proportion of patients with adequate relief of overall IBS symptoms	0.01
Zeng J et al, 2008(227)	<i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i> , <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium Longum</i>  Fermented milk, 200g BD for 4 weeks (each ml contained at least $1.3 \times 10^8$ CFU total)	IBS-D (Rome II)	Decreased small bowel permeability  Primary end points not GI symptoms.	-

(Adapted from Hungin AP. Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice - an evidence-based international guide. *Aliment Pharmacol Ther* 2013(224))

Given this data, probiotics may be used in IBS to improve global symptoms, bloating and flatulence however recommendations with regards to specific preparations are unable to be made due to insufficient evidence(194).

*f) Non-absorbable Antibiotics (Rifaximin)*

Modifying the intestinal microbiota is a growing area of interest in the management of IBS. The role of antibiotics in IBS has already demonstrated encouraging results. Eradication of small intestine bacterial overgrowth in IBS patients with antibiotics was shown to improve symptoms of abdominal pain and diarrhoea and eliminate IBS in approximately 50% of patients(228). Normalisation of the lactulose breath test in IBS patients with Neomycin has resulted in correction of bowel habit in 35% of patients(229).

Rifaximin is derived from Rifampin through the addition of a pyridimazole ring to minimize systemic absorption and is a semi-synthetic antibiotic(230). It acts by inhibiting synthesis of bacterial ribonucleic acid (RNA), through binding to the  $\beta$  subunit of the bacterial deoxyribonucleic acid dependent RNA polymerase(231). The anti-bacterial effects include activity against gastrointestinal aerobic and anaerobic Gram-positive and gram-negative bacteria, while imposing a relatively low risk of bacterial resistance(232-234). Rifaximin may also alter the function of the intestinal microbiota through a number of mechanisms. Modification of bacterial adherence may occur through preventing mucosal inflammation in reducing mucosal bacterial colonization and infection. Bacterial metabolism may also be altered as well as down-regulation of bacterial virulence(235).

Due to limited evidence for its use, Rifaximin is only indicated in IBS-D and in not other IBS subtypes(156). The TARGET study group have demonstrated in two phase 3, double-blind, placebo-controlled trials (TARGET 1 and TARGET 2) that patients with IBS without constipation, treatment with 550mg Rifaximin for two weeks provided significant relief of global IBS symptoms, including abdominal pain, bloating and loose or watery stools(236). The TARGET 3 study, which is currently only published in abstract form, showed that in patients with IBS-D who initially responded to Rifaximin and then had symptom recurrence, were then given a repeat course of Rifaximin. This resulted in significant improvements in IBS related abdominal pain and stool consistency compared with patients receiving a



placebo(235).

*g) Faecal Microbiota Transplant (FMT)*

This involves administering healthy donor stool into the colon of symptomatic patients. FMT has already demonstrated therapeutic potential in *Clostridium difficile* infection and inflammatory bowel disease (IBD)(237).

## **Fermentation: *Short chain fatty acids, the gut microbiome and volatile organic compounds***

Our research group has coined the term 'fermentome' to express the complex relationship between diet (resulting in the production of short chain fatty acids), the intestinal microbiota and volatile gases. It has been proposed that the gas products of fermentation serve as biotransmitters and are important for intestinal homeostasis through intestinal microbiota chemical signaling(238).

The human right colon is well suited as its role as a fermentation chamber, with its ordered inlet in the form of the ileo-caecal valve and no corresponding valve at the outlet (i.e. the hepatic flexure). The hepatic flexure is acutely angled, connected to the diaphragm via the colophrenic ligament and partially rotated. Contraction of the teniae coli (the longitudinal strap muscles across the hepatic flexure) augments the 'kink', which creates a closed chamber for fermentation to occur. Relaxation of the teniae coli allows for the chamber to open and its contents to transit through the colon(238).

## **1.3 Short Chain Fatty Acids (SCFAs)**

### **1.3.1 Overview of SCFAs**

SCFAs are the end products of metabolism by anaerobic microbes in the colon and have been proposed to be the link between microbiota and host tissues(239). Faeces consist predominantly of water (80-90%) with its fibrous residue being mainly in the form of non-starch polysaccharides (NSP)(238). NSPs are the main substrates for fermentation by resident intestinal bacterial digestive enzymes, resulting in the principal SCFAs of acetate, propionate and butyrate, which contribute to normal bowel function and are an energy source for the colonocytes, as well as yielding metabolisable energy for microbial growth(238, 240). If the capacity of the colonic microbiota to metabolise the polysaccharides is exceeded, the result may be an osmotic diarrhoea(241). The minor branch chained SCFAs (iso-butyric, iso-valeric and n-valeric acid) found in small amounts in faeces are derived from bacterial fermentation of proteins. Mucus, sloughed cells and gastrointestinal secretions may also contribute to the production of SCFAs(241).

SCFAs are weak organic fatty acids, categorized by the number of their carbons. They refer to carboxylic acids with aliphatic tails of 1 to 6 carbons, including acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and hexanoate (C6)(241, 242). The proportions of SCFA in the lumen are approximately 60% acetate, 25% propionate and 15% butyrate with the normal concentration range of these SCFAs being 70-100mM (241, 243). Rodent studies demonstrate that the production of propionate and butyrate are inversely related. Sources of fibre (such as oat bran) enhance steroid excretion, which lowers plasma cholesterol and increases the contribution of propionate relative to butyrate, whereas wheat bran has the opposite effect(244).

An estimated 100-200 mM of SCFAs are produced daily, 95-99% of which, are absorbed by the colon(241, 245). This absorption is associated with

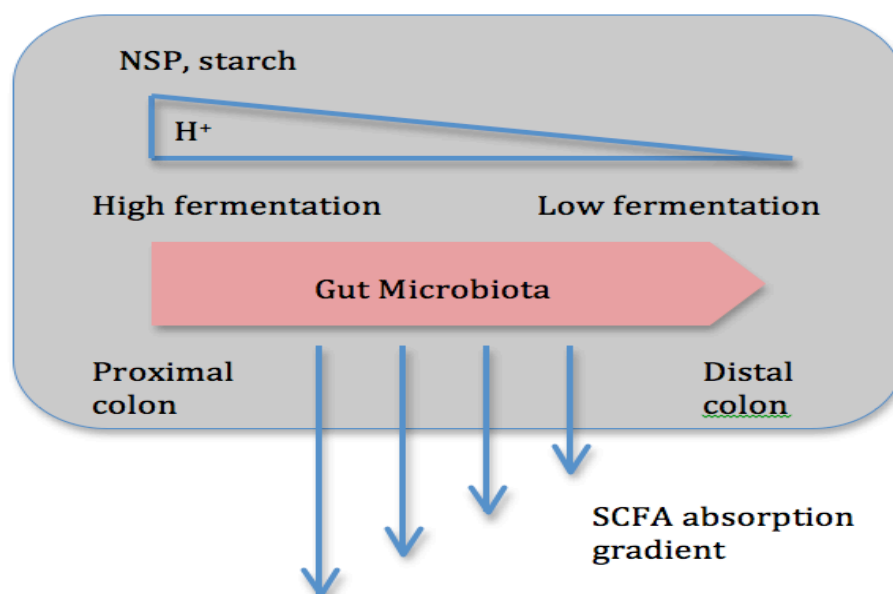
increased sodium absorption and bicarbonate secretion. The equilibrium of ionised and protonated SCFAs and their transport across cell membranes are dependent on pH. The transport of other solutes and electrolytes in the colon is altered by absorption of SCFAs. This may occur through formation of isolated pH compartments in the crypts, modifying transfer of weak electrolytes and acid-base transport(241). In rodent distal colons, SCFAs have demonstrated inhibition of cAMP-mediated  $\text{Cl}^-$  secretion(246). Therefore, SCFAs possess both anti-secretory and pro-absorptive properties(241). Following a right hemicolectomy, the effect of SCFAs stimulating sodium and water absorption is maintained through adaptation of the remaining left hemicolon. However, after a total or subtotal colectomy, the effect is lost and diarrhoea occurs secondary to impaired fermentation(246).

Fermentable carbohydrates are able to modify the microbial ecology either by supplying SCFAs or acting as substrates. The principal products of fermentation in the adult human are heat, gases ( $\text{CO}_2$ ,  $\text{CH}_4$ ,  $\text{H}_2$ ) and SCFAs(240). There are three bacterial reactions consuming a large proportion of the gas volume in the colon. Firstly, methanogenic bacteria, accounting for 50% of the population, reduce  $\text{CO}_2$  to  $\text{CH}_4$  and consume  $\text{H}_2$  in the process. Secondly, sulphate-reducing bacteria that reduce  $\text{SO}_4$  to sulphides, including  $\text{H}_2\text{S}$ . Thirdly, acetogenic bacteria consume variable amounts of  $\text{H}_2$  and reduce  $\text{CO}_2$  to acetic acid(241).

Fermentation predominantly occurs in the proximal large bowel and SCFAs are transported in the faecal stream to distal regions with a higher concentration and a progressive decline of SCFAs being observed along the bowel (figure 1)(240). This is supported by levels of SCFA excretion being greater with transverse colostomy compared to sigmoid colostomy with concentrations of levels in sigmoid colostomy fluid being 40-50% of those with a transverse colostomy(247). Depending on diet, the proximal colon assumes a total concentration of SCFAs of 70-140 mM, reducing to 20-70mM in the distal colon. The total SCFA concentration or the individual acids observed in the distal colon is not predictive of those found proximally.

Numbers of indigenous bacteria are also greatest in the proximal colon(240). The luminal pH in the proximal colon is lower due to the increasing concentration of acidic fermentation products(243). This reduced pH encourages the formation of butyrate through the acidic pH allowing the butyrate-producing bacteria to compete with Gram-negative carbohydrate-utilising bacteria, such as *Bacteroides* spp(248). In addition, *in vitro* studies have observed that lower pH values (and increased concentrations of SCFAs) are recognised to prevent the overgrowth of pH-sensitive pathogenic bacteria(249).

**Figure 1: Fermentation in the colon**



The rate of transit of colonic contents, as well as the type of fibre determines the rate and degree of fermentation(240, 241). Water-soluble fibre such as pectin is virtually completely fermented by bacteria whereas only 5-20% of cellulose and lignin undergo anaerobic fermentation in the colon. Diets high in resistant starches, fibre and complex carbohydrates result in increased production of SCFAs(241). Foods with greater quantities of soluble NSP undergo the greatest losses on transit and this is accompanied by increased bacterial faecal excretion(250, 251). At whole gut transit times of greater than 50 hours, butyrate cannot be detected and this is likely due to colonic uptake(240). A significant difference in the total SCFA concentration as well

as concentrations of the individual acids (acetic, propionate and butyrate) between men and women is observed with higher concentrations being evident in men. Concentrations of the minor acids are similar between the sexes(252).

### **1.3.2 SCFAs and Intestinal Health**

Once absorbed by the colonocyte, SCFAs are used locally as fuel for the colonic epithelial cells with butyrate being the preferred fuel resulting in 70-90% being metabolized by the colonocyte. SCFAs provide 5-10% of human basal energy requirements(239). Butyrate is favorably used over propionate and acetate in a ratio of 90: 30: 50(241). Propionate is a substrate for hepatic gluconeogenesis and inhibits cholesterol synthesis in the liver(241, 253). Acetate is used in the production of long chain fatty acids, glutamate, glutamine and beta-hydroxybutyrate(253). SCFAs also modulate secretion of the satiety hormones, including leptin and peptide YY(254, 255).

Gram-positive anaerobic bacteria have the ability to produce butyrate with the two main producers being *Eubacterium rectale/Roseburia* spp of Clostridial cluster XIVa of the Firmicutes and *Faecalibacterium prausnitzii*, of the Clostridial cluster IV cluster(256). The pH of the colon may be lowered by the production of SCFAs. This is of significance due to the varied tolerance of a reduced pH for different species within the Firmicutes phylum with the butyrate-producing associated Clostridium cluster IV growth rates being considerably reduced while the Clostridium cluster XIVa group demonstrated the smallest reduction in growth rate(257).

#### ***Pro-absorptive/Anti-secretory Effects***

SCFAs, in particular butyrate, exert regulatory pro-absorptive/anti-secretory effects on the transepithelial ion transport and this occurs through various mechanisms. Stimulation of NaCl absorption occurs via two coupled transport systems ( $\text{Cl}^-/\text{HCO}_3^-$  and  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{butyrate}$  and  $\text{Na}^+/\text{H}^+$ ) on the colonic brush border.  $\text{Cl}^-$  secretion inhibition occurs through blockage of Na-K-2Cl, a cotransporter on the enterocyte basolateral membrane(243). Butyrate therapy, in the form of resistant starch (a precursor of butyrate) has

demonstrated an improvement in stool volume and recovery time in children with cholera diarrhoea(258, 259).

### *Trophic Effects*

Muscular atrophy of the colon may occur in diets lacking fibre. DNA synthesis and content as well as mucosal weight increase with fibre and this effect is mediated by SCFAs, which is considered to have a trophic effect on normal colonic mucosa(245). Rodent studies where subjects were fed a fibre-free diet but intracolonicallly infused with either a combination of SCFAs or a butyrate solution demonstrated significant colonic growth as reflected by increased protein, mucosal mass, RNA and DNA(260). SCFAs have also been observed to mediate colonotrophism locally in stimulating cell proliferation in the basal 60% of the crypts(261). This may be due to increases in visceral blood flow, aerobic oxidation of SCFAs for energy, stimulation of the enteric nervous system and increased production of enterotrophic hormones(262). SCFAs have been shown to increase blood flow in the rectum(263). Rectal infusion of SCFAs into surgical patients demonstrated a 1.5-5 fold increase in splanchnic blood flow(264). This is thought to be secondary to chemoreceptors, local neural networks as well as direct effects on smooth muscle cells. It is assumed that greater blood flow improves tissue oxygenation and transport of absorbed nutrients(240).

### *Anti-carcinogenic Effects*

The anticarcinogenic effects of butyrate have been well studied. Dietary fibre is well documented in several epidemiological studies in protecting against colorectal cancer(265-268). *In vitro*, the addition of butyrate to carcinoma cell lines resulted in induction of apoptosis, inhibition of proliferation and differentiation of tumour cells(269-271). Apoptosis is enhanced by butyrate and not by the p53 gene, which controls programmed cell death and is often seen in tumours(272, 273). Propionate and acetate are also capable of inducing apoptosis but to a lesser extent than butyrate and require higher concentrations to do so ( $\geq 40\text{mM}$ )(274). At concentrations of 1-5 mmol/L, butyrate can switch the phenotype of cancer cells to being

more consistent with normal cells and restoration of the cytoskeleton and composition of the glycosylated cell surface components. At the molecular level, butyrate has been observed to modify oncogene expression (downregulation of N-ras) and induce hyperacetylation of histones(275, 276).

#### *Immunity Effects*

SCFAs have been acknowledged as potential mediators involved in the effects of the intestinal microbiota on intestinal immune function through acting on endothelial cells and leucocytes by two mechanisms. These are inhibition of histone deacetylase and activation of G-protein-coupled receptors - GPCRs (GPR41 and GPR43). They regulate various leucocyte functions including production of eicosanoids, cytokines (TNF- $\alpha$ , IL-2, IL-6 and IL-10) and chemokines (MCP-1 and CINC-2). SCFAs also affect the ability of leucocytes to migrate to the foci of inflammation and destroy microbial pathogens(239).

#### *Biological Effects*

SCFAs play an important role in the maintenance of a functioning gastrointestinal system by slowing the passage of food in the upper gastrointestinal tract to improve nutrient digestion(240). After a rectal infusion of SCFA or ingestion of lactulose (fermentable carbohydrate), a reduction in gastric tone, giving an expansion of volume was observed. The mechanism of action is the activation of the ileocolonic brake in a dose-dependent fashion(277).

The biological effects of SCFAs on the colon have been implicated in intestinal disease, including diversion colitis and ulcerative colitis. It was hypothesised that diversion colitis arose from a deficiency in SCFAs and instillation of a SCFA solution resulted in an improvement in the endoscopic score, resolving the inflammation(278). However, this finding was contradicted by another study, which did not identify an improvement in either endoscopic or histological appearance in these patients when given a



butyrate enema(279). Impairment in butyrate oxidation in isolated colonocytes in patients with both active and quiescent ulcerative colitis has been observed(280). Oxidative stress plays a role in inflammation and the initiation and progression of carcinogenesis(281, 282).

Butyrate regulates the colonic defense barrier, where intestinal permeability is decreased with a reduction in its concentration(283, 284). Butyrate participates in the assembly of tight junctions via AMP-activated protein kinase, which affects the intestinal wall permeability(285). In colonocytes, butyrate has been observed to increase expression of the MUC2 gene, inducing mucin synthesis, which can affect the mucous layer resulting in improved protection against luminal agents(286, 287).

A rodent study has exhibited the ability of SCFAs in regulating the ENS and colonic motility. Rats given a resistant starch diet demonstrated increased colonic transit with butyrate increasing the cholinergic-mediated colonic circular muscle contractile response *ex vivo*(288). Butyrate may affect colonic visceral perception as administration of butyrate enemas in healthy volunteers revealed an increase in colonic compliance and decrease in pain, urge and discomfort measured with a rectal barostat procedure(289).

### **1.3.3 SCFAs in IBS and BAD**

There are very few studies, which examine the role of SCFAs in IBS and none to date in BAD. An association between the gut microbiome and IBS has been observed in several studies with inconsistent results in characterising the microbiota in IBS. It is crucial to consider that the function of the microbiota may be as important as the phylotype in the maintenance of health and genesis of disease. Function may be measured as microbial metabolites in the form of SCFAs.

Studies have demonstrated conflicting evidence with one study reporting reduced SCFAs in IBS-C and increased SCFAs in IBS-D(290). Another study observed reduced SCFAs in IBS-D(291). The production of SCFAs is

able to lower the pH of the colon(257). A study observed a reduction in the proportion of *Bacteroides* in patients with IBS and *Bacteroidetes* are recognised to be inhibited in acidic conditions(257, 292). The same study reported an increase in *Bifidobacterium adolescentis* in IBS although this species has not been found to have a reduction in its growth rate in acidic conditions(292, 293). Members of the *Bacteroides* genus are saccharolytic commensals and produce acetic, succinic, lactic and propionic acids(294). Bifidobacteria produce acetic and lactic acids with smaller quantities of formic acid and ethanol(295). Depending on the dysbiosis observed, one would expect varying concentrations of the different SCFAs produced.

In the quest to demonstrate altered gut microbiota with a relationship between IBS symptoms and an unbalanced organic acid (SCFAs) profile, a study revealed significantly higher counts of *Lactobacillus* and *Veillonella* in patients with IBS. Compared to healthy controls, these patients also expressed significantly increased levels of acetic acid, propionic acid and total organic acids. Although the quantity of bowel gas between the two patient cohorts did not reach a statistical difference, significantly worse gastrointestinal symptoms, quality of life and negative emotions were observed in IBS patients with high acetic or propionic acid levels(296). The association observed between organic acids and visceral IBS symptoms is reflected in a rodent study where rats who were treated with an intracolonic infusion of acetic acid demonstrated higher sensitivity to colorectal distension than those infused with saline(297). The heterofermentative species of *Lactobacillus* metabolises lactic and acetic acid from fructose or glucose(298). *Veillonella* is able to produce acetic and propionic acid from lactic acid therefore the altered gut microbiota profile observed appears to be associated with higher levels of organic acids in IBS patients(299). This notion is further supported by a study of patients with small bowel bacterial overgrowth who had a 4-fold increase in the total concentration of SCFAs in their jejunal secretions compared to their healthy counterparts. These patients display a colon-like microbiota in the small intestine with the modified microbial composition accounting for most of the SCFAs in the

jejunal secretions(300). In addition, the use of antibiotics has demonstrated a reduction in the total concentration of SCFAs in faecal excrement(301).

As well as the disease itself, treatment for the condition may further alter the intestinal microbiota and production of SCFAs. IBS-D and IBS-M patients who were put on a low FODMAPs diet (LFD) demonstrated a reduction in *Clostridium*, *Megasphaera*, *Pediococcus*, *Actinobacteria*, *Bifidobacterium*, and *Faecalibacterium prausnitzii* bacteria as well as a concentration of total SCFAs and n-butyric acid. Interestingly, after ten days of supplementation with a high-fructo-oligosaccharides (FOS) diet, the levels of these bacteria increased but the SCFAs remained unchanged. *Clostridium*, *Megasphaera*, *Pediococcus* and *Faecalibacterium prausnitzii* are members of the Firmicutes phylum, which is known to contain several fermenting bacteria(302). *Faecalibacterium prausnitzii* in particular, is a major butyrate producing commensal and has been referred to as a biomarker of intestinal health in adults(303). Therefore, a reduction in SCFA production is to be expected, especially if other bacteria increase in numbers and occupy their metabolic niches(302). Diminished levels of *Bifidobacterium* and *Faecalibacterium prausnitzii* with a LFD are likely secondary to the reduced availability of galacto-oligosaccharides and prebiotic fructans (including FOS)(304). This belief is supported by the fact the study reported a rapid diet-induced reversal of the gut microbiota with FOS supplementation.

A recent study reported that the difference between faecal propionic and butyric acid (mmol/l) was the best diagnostic biomarker for all subgroups in IBS, with a cut-off value >0.015 mmol/l indicating IBS(305).

## 1.4 The Gut Microbiome

There are three terms used when studying the intestine as a habitat. The first term is 'microbiota' which describes the microorganisms present in a defined ecosystem in the body(306). The second is 'metagenome', reflecting the assembly of genes and genomes of the microbiota, providing sequence information. Lastly, the term 'microbiome' defines the habitat with its microbial population, their genes and genomes and the interactions between themselves and with the host.

### 1.4.1 Overview of the Gut Microbiome

The gastrointestinal tract harbors a diverse community of approximately  $10^{14}$  microorganisms comprising of 500 to 1000 distinct bacterial species and is the most heavily colonized organ in the human body(18). There is 10 times the quantity of commensal bacteria and fungi inhabiting the human body compared to human cells(307). The intestinal microbiota are not homogenous and are distributed along the length of the whole gastrointestinal tract, with the diversity and density increasing from the stomach to the colon(307). A continuum arises from  $10^1$  to  $10^3$  bacteria/gram of contents in the stomach and duodenum to  $10^4$  to  $10^7$  bacteria/gram in the jejunum and ileum, culminating in  $10^{11}$  to  $10^{12}$  bacteria/gram in the colon(308). *Firmicutes* and *Actinobacteria* of the Bacilli class enrich the small intestine whereas anaerobic bacteria constitute the majority of the colonic gut microbiota and are predominantly represented by *Bacteroidetes* and the *Firmicutes*, with *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and *Cyanobacteria* being present in minimal proportions(309, 310).

As well as the longitudinal variation exhibited by the intestinal microbiota, there is also the presence of latitudinal heterogeneity in the composition of the microbiota. The intestinal lumen is separated from the epithelium by a thick, complex mucus layer. The communities of microbes present in the intestinal lumen which are either dispersed in liquid faecal matter or bound

to food particles, vary significantly from the mucosa-associated microbiota embedded and attached to this mucus layer, as well as the microbiota that appear in immediate proximity of the epithelium(311). The gastrointestinal tract is constituted mainly by the luminal bacteria, which are important in intestinal homeostasis, with the toxins and metabolites of these bacteria modulating the host immune system(312, 313). Several bacterial species, including *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* have been observed in faeces but did not access the mucus layer and epithelial crypts, where *Clostridium*, *Lactobacillus*, and *Enterococcus* have been found in the small bowel(314).

Over a lifetime, the composition of the gut microbiome changes significantly and age-specific alterations imply the microbiome-mediated effects on health of the host. Initial colonization of the gastrointestinal tract may occur even prior to birth with the detection of bacteria in the placenta, amniotic fluid, cord blood and foetal membranes suggesting that the presence of these microorganisms may not necessarily indicate a sterile *in utero* environment or pathological state(315). Vaginal microbial abundance and diversity are reduced in pregnancy, with the dominance of *Lactobacillus* species being observed(316). Given the vagina's close proximity to the foetus, it would be assumed as the primary source of inoculum however, the placental microbiome has been demonstrated to be more akin to the human oral microbiome, composed of nonpathogenic commensal microbiota, including the *Firmicutes* and *Tenericutes* phyla(317). Bacteria commonly enter the circulation through ulcerated gingival crevicular tissue surrounding the teeth and this is considered to be the source of bacterial translocation for physiological and pathological bacteria from the oral cavity, which then colonise the placenta and are exposed to the foetus. This is reflected in the fact of preterm birth being associated with both periodontal disease and intrauterine infection(315, 318). Throughout childhood and adolescence, the maturation of the intestinal microbiota appears to parallel the physiological development central nervous system and gastrointestinal tract(319, 320). Once established, the intestinal microbiota within individuals remains

relatively stable over time however, there is extensive variability between individuals(321, 322).

The composition of the microbiota is unique to each individual and it has been demonstrated that the faecal microbiome of identical twins share less than 50% of species phylotypes(323). In the elderly population, there are age-related physiological changes in the gut microbiota, which may result in a microbial imbalance due to chronic low-grade inflammation(324, 325). High throughput sequencing analysis has demonstrated a differing composition of gut microbiota of older people (above 65 years) compared to younger people with a predominance of the phylum *Bacteroidetes*(326). *Bacteroidetes* have been identified primarily in residents in long-stay care environments and these individuals demonstrated considerably less diverse microbiota with loss of the community-associated flora, which was associated with frailty(327).

Environmental changes may account for the differences in intestinal microbiota at the same anatomical site at various points in time(328). This includes dietary changes, use of antimicrobials, vaccination and sanitation. The strong impact of diet on the intestinal microbiota is already demonstrated before weaning in babies. The microbiota of breastfed babies is less diverse than formula-fed babies, with a predominance of *Bifidobacterium* spp(329, 330). This is assumed to be the effect of human milk oligosaccharides serving as metabolic substrates to only a limited number of bacteria(331). With the introduction of solid foods, a shift towards an adult-like microbiota is observed(332). Variations in geographical location impact on the composition of the intestinal microbiota. Non-Western populations, who consume a diet high in plant-rich carbohydrates, have been observed to have a more diverse microbiome enriched in *Prevotella* spp, at the expense of *Bacteroides* spp, compared to a Western diet high in animal protein, fat, sugar and starch(333). A diet high in resistant starch has demonstrated an increase in *Eubacterium rectale* and *Ruminococcus bromii* species, which are recognized for their saccharolytic properties(334). Increased protein and animal fat in the diet has been associated with the

*Bacteroides* enterotype whereas a diet enriched in carbohydrates is associated with the *Prevotella* enterotype. In addition, obese individuals demonstrate an increase in Firmicutes and a decrease in Bacteroidetes, which is likely secondary to differences in diet(335). Short-term modifications in diet appear to only have a modest change in the microbial composition(122, 336).

Table 5 below demonstrates factors, which influence the intestinal microbiome at different life stages.

**Table 5: Life stages factors influencing the intestinal microbiome**

Life Stages	Age Specific Factors	Generic Factors
<b>Prenatal</b>	Overall health status (e.g. maternal stress, pregestational diabetes)	Diet
	Prenatal probiotics supplementation, maternal diet	
<b>Neonate</b>	Gestational age at delivery	Antibiotics
	Mode of delivery (vaginal delivery vs C-section)	
<b>Infant</b>	Breast milk vs formula, antibiotic exposure	Genetics
	Familial environment vs daycare, pets, siblings	
<b>Toddler</b>	Familial environment/daycare, siblings	Geographical location
<b>Adolescent</b>	Hormone levels, ethnicity	
	Life events (e.g. pregnancy)	Social context
<b>Adult</b>	Obesity, lifestyle (changes in diet) and overall health status (infection/antibiotic use)	
	Urban vs rural environment	
<b>Geriatric</b>	Nursing home vs community living	
	Decline in health, impaired dentition, fraility	

(Adapted from Greenhalgh K et al. The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. Environ Microbiol 2016(315))

Alterations of the intestinal microbiome have been implicated in various gastrointestinal diseases including, IBS, *Clostridium Difficile* infection and IBD(306).

#### **1.4.2 Functional Aspects of the Gut Microbiome**

The gastrointestinal tract has a surface area of around 32 square metres and is the largest human interface with our environment(337). This complex interface forms the mucosal barrier and is composed of the microbial flora, soluble anti-microbial molecules, mucus, epithelial layer, the intestinal lamina propria containing connective tissue and immune cells, intraepithelial compartment and mucosa-associated lymphoid tissue. The mucosal barrier plays the vital role of protecting against environmental threats and promoting nutrient absorption(338).

##### *Physiological Functions*

The gut microbiota contains at least 100 times as many genes as the human genome, most of which confer physiological functions. These recognized roles include metabolic functions such as vitamin synthesis, regulating the uptake and deposition of dietary lipids, absorbing indigestible carbohydrates and modulating the intestinal epithelium's absorptive capacity for optimum nutrient metabolism. Colonic bacteria, including *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Fecalibacterium*, and *Enterobacteria* ferment carbohydrates and indigestible oligosaccharides to produce SCFAs. These are mainly composed of butyrate, propionate and acetate, which confer a rich energy source for the host(339, 340). *Bacteroides* are the main organisms responsible for the metabolism of carbohydrates and perform this by expressing enzymes such as glycoside hydrolases, glycosyl transferases and polysaccharide lyases(341). This is important as the production of butyrate prevents the accumulation of toxic metabolic by-products such as D-lactate(342). Metabolism of proteins is conducted via amino acid transporters located on the bacterial cell wall. This facilitates entry of amino acids from the intestinal lumen into the bacteria, where gene products convert them into antimicrobial peptides (AMPs - bacteriocins) and small signaling molecules(341). Suppression of the inhibition of lipoprotein lipase



(LPL) has been observed by the intestinal microbiota with increased LPL activity in adipose tissue encouraging fatty acid uptake into adipocytes(343).

### *Protective functions*

Protective functions incorporate the maintenance of intestinal barrier integrity and barricading against invading pathogens by competitive exclusion through production of antimicrobial peptides, engagement of attachment sites and consumption of nutrient supplies(311). The presence of commensal bacteria and products of bacterial metabolism allows for induction of the expression of AMPs and their activation to protect against invading pathogens and prevent overgrowth of the indigenous bacteria themselves(311). Paneth cells, which are located at the base of small intestinal crypts, express a variety of AMPs, which is mediated by the presence of normal intestinal microbiota(344, 345). They also produce matrilysin, a matrix metalloproteinase that activates defensins (an AMP). Defensins are produced in their inactive form (prodefensins), which require proteolytic cleavage for activation. Colonisation of *B. thetaiotaomicron* in germ-free mice has been observed to induce matrilysin expression(346). SCFAs and lithocholic acid, which are bacterial metabolic products, induce the expression of cathelicidin (AMP) by mechanisms involving histone deacetylation and the mitogen activated protein kinase/extracellular signal regulated kinases pathway(347-349).

Members of the genus, *Lactobacillus*, produce lactic acid, which not only provides an inhibitory environment for the growth of many bacteria, but also potentiates the antimicrobial effect of lysozyme in disrupting the bacterial cell wall membrane(350). This specific genus also generates antimicrobial substances that are active against a wide breadth of both gram negative and positive enteropathogenic bacteria(351).

Various microbiota contribute to the maintenance of the intestinal epithelium barrier integrity, including *B. thetaiotaomicron*, which has demonstrated induction of the expression of small proline-rich protein 2a (sprr2a), a protein important in desmosome maintenance(352). Several probiotic

strains of *Lactobacillus*, have been observed in helping maintain tight junctions within the intestinal wall, protecting against intestinal injury or pathogen attack(353). In vivo, microbial cell wall peptidoglycan principally stimulates signaling via TLR2, which has been shown to maintain tight junctions and decrease apoptosis, thus contributing to the integrity of the intestinal epithelium(354). Microbial regulation of angiogenesis, which contributes to the structural development of the intestinal mucosa has been observed by indigenous bacteria inducing Transcription factor angiogenin-3, a protein implicated in the development of intestinal microvasculature(355).

#### *Immunomodulation Functions*

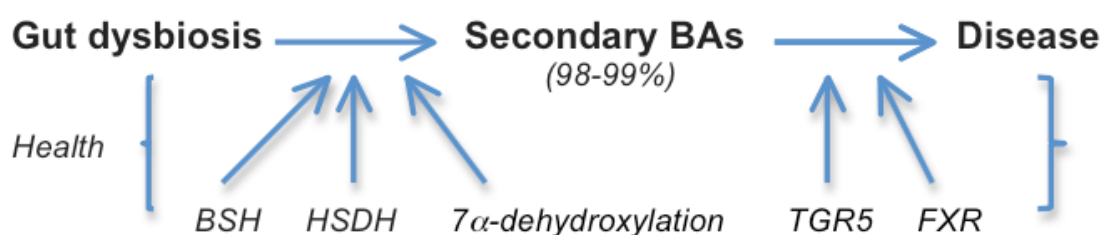
Immunomodulation functions include tolerance to dietary and microbial antigens. This is mediated by the induction of regulatory T cells as well as inhibiting overgrowth of the gut microbiota and translocation to systemic sites through activating intestinal dendritic cells (DCs), which selectively induces the production of IgA from plasma cells. Despite infiltration of the lamina propria with activated immune cells and only a single epithelial layer allowing for separation from the gut microbiota, healthy individuals do not demonstrate pathological features. Therefore, regulatory mechanisms exist to ensure intestinal immune homeostasis in a healthy gut but to also stimulate a protective immune response in the presence of pathogen invasion. Small numbers of live commensal organisms penetrate the Peyer's patches and the bacterial antigens are taken up by the DCs resulting in mucosal immune responses and induction of IgA B cells. These B cells occupy the lamina propria by recirculating through the lymph and bloodstream to secrete protective IgA. This safeguards against mucosal penetration of bacteria as the DCs loaded with bacteria are confined to the mucosal immune compartment by the mesenteric lymph nodes, ensuring local induction of immune responses to the bacteria while the systemic immune system remains relatively ignorant of these organisms(356-359).

#### **1.4.3 The Gut Microbiome, Dysbiosis and Bile Acid Signalling**

Dysbiosis occurs when pathological imbalances in gut bacterial colonies

precipitate disease and has been linked to the dysmetabolism of BAs in the gut(360). Figure 2 below illustrates a proposed relationship between gut dysbiosis, modified BA pool and disease.

**Figure 2: Proposed schema of interplay between gut dysbiosis, modified BA pool and disease.**



In health, secondary BAs are modified by microbial BSH and HSDH enzymes through deconjugation, oxidation and epimerization as well as dehydroxylation via 7 $\alpha$ -dehydroxylation activity. The BA metabolites as a result of microbial transformations act as signaling molecules via the TGR5 and FXR receptors to regulate intestinal homeostasis. In disease, it is unclear how gut dysbiosis causes a modified BA pool, which then results in disease, which is possibly secondary to impaired BA signaling. (BSH – bile salt hydrolases; HSDH - hydroxysteroid dehydrogenases; TGR5 – G protein coupled BA receptor; FXR – farnesoid x receptor)(360)

### **BA Signaling**

As a result of microbial transformations, BA metabolites act as signaling molecules and through activation of the BA activated receptors TGR5 and FXR, have demonstrated regulation of intestinal homeostasis by inhibiting inflammation, preventing pathogen invasion and maintaining cell integrity. In the intestine, TGR5 is expressed by enteric neurons and enterochromaffin cells and is principally activated by secondary BAs, including DCA and LCA to release glucagon-like peptide 1 and 5-hydroxytryptamine(361, 362). The receptor TGR5 has been shown to mediate the prokinetic effects of intestinal BAs with faster colonic transit and increased defecation frequency observed in TGR5-transgenic mice(363). In patients with IBS-D, TGR5 single nuclear polymorphism has been associated with faster small bowel transit time(364). TGR5 has been found to regulate intestinal barrier integrity as TGR5 (-/-) mice have demonstrated a modified architecture of epithelial tight junctions with increased expression and abnormal subcellular

distribution of zonulin-1, a tight junction protein. This resulted in increased intestinal permeability and susceptibility in developing dextran sulphate sodium (DSS) induced colitis in rodent models(365). TGR5 also minimizes production of pro-inflammatory cytokines (IL-1 $\alpha$ , IL-2 $\beta$ , IL-6 and TNF $\alpha$ ) stimulated by lipopolysaccharides in macrophages and Kupffer cells through inhibition of NF-kB(366).

#### *Physiological Effects of BA Signalling*

The BA receptor FXR has been implicated in participating in barrier function and immune regulation of the intestine. In vivo and in vitro models have demonstrated an FXR agonist inhibiting an inflammatory cytokine (TNF-) secretion in different human immune cell populations and preventing chemically induced intestinal inflammation, with improvement of colitis symptoms, reduced goblet cell loss and inhibition of epithelial permeability in wild-type mice(367). Intestinal FXR has a key role in limiting bacterial overgrowth and translocation in the distal small intestine with resultant disruption to the gut epithelial barrier through the regulation of several genes, including Ang1, iNOS and IL18, which have recognized antimicrobial actions(368). The cytokine IL-18, which is induced by FXR activation, stimulates defense and resistance to a breadth of pathogens, including intra- and extracellular bacteria and mycobacteria(369).

#### *The Relationship between Dysbiosis and BAs*

The degree of activation of BA receptors is influenced primarily by the gut microbiota and therefore dysbiosis may result in abnormal BA modification resulting in the development of gastrointestinal disease. It is hypothesized that BA modification is shared by a variety of bacterial species/genus and that an increase in some species may be associated with the inhibition of other species involved in BA metabolism(370). Through expansion of regulatory T cells or downregulation of inflammatory cytokines, *Faecalibacterium*, *Roseburia*, *Lactobacillus*, particular *Clostridial* clusters and *Bacteroides fragilis* have been associated with reduced intestinal inflammation. These bacteria have a role in BA deconjugation and may be lost in IBD, especially Crohn's disease(338, 371-373). Intestinal dysbiosis

has been associated with BA dysmetabolism in patients with IBD, indicated by the observation of a reduction in faecal secondary BA concentrations and an increase in sulphated forms. These forms were particularly increased in active IBD patients (assessed by disease activity indices). In vitro, secondary BAs exerted anti-inflammatory effects, whereas sulphation of the secondary BAs eliminated their anti-inflammatory properties, signifying that dysbiosis in IBD led to impaired microbiota enzymatic activity. This in turn modified the BA pool composition, resulting in disease(374).

The inverse, where BAs may influence the composition of the intestinal microbiota may also be true. BAs have direct antimicrobial action on intestinal microbes, with DCA being more potent than CA, due to its hydrophobicity, which increases its affinity for the phospholipid bilayer of the bacterial cell membrane(375). They also have indirect effects via FXR-induced AMPs(376). A rodent study demonstrated rats who were fed a diet of CA, had phylum-level modifications of their intestinal microbiome, with an expansion of *Firmicutes* at the expense of *Bacteroidetes*, with outgrowth of several bacteria in the classes *Clostridia* and *Erysipelotrichi*(377). Hydrolysis of conjugated BAs by BSHs yields free BAs, which become weak acids and in the neutral physiological pH range where most of the BAs are in a non-ionised form, provides them with strong bactericidal activity. The hydrophobicity of these molecules is further increased by the 7 $\alpha$ -dehydroxylation reactions by the intestinal microbiota in the large intestine, which eliminates the functional hydroxyl group at C-7<sup>386</sup>. The bactericidal activity of the BAs increases as the molecules are transported from the duodenum to the distal colon. Increased levels of BAs in the gut favour Gram-positive members of the *Firmicutes*, including 7 $\alpha$ -dehydroxylating bacteria. Reduced levels of BAs appear to favour Gram-negative bacteria, some of which produce potent lipopolysaccharide (a component of the cell wall) and include potential pathogens(378).

#### ***BA Modification and Disease***

In IBS-D patients, modifications in faecal BA composition have demonstrated a significant increase in primary BA and a parallel decrease in

secondary BA compared to healthy controls. In this study, this observation correlated with a higher stool frequency and reduced stool consistency. This may reflect the influence of dysbiosis found in this study with IBS-D patients demonstrating a reduction in *bifidobacterium*, an increase in *E. coli* and decreased counts of *leptum*. The *leptum* group encompasses many bacteria (in particular *Ruminococcus* and *Clostridia*) that are involved in BA transformation therefore reduced numbers of these bacteria may account for the decreased transformation activity of the microbiota, resulting in increased primary BAs and reduced secondary BAs(370, 371). This would suggest that the altered gut microbiome is the primary driver for BA dysregulation in IBS. Results from this study are supported by another randomized controlled study where CDCA, a primary BA, was given to healthy subjects and was found to significantly accelerate colonic transit, increase stool frequency and decrease stool consistency(72). A recent study supports this hypothesis with IBS-D patients being found to have increased faecal CDCA, compared to healthy controls and patients with IBS-C(370). At the intestinal mucosal level, CDCA inhibits water absorption, thereby inducing watery diarrhoea, as seen in patients with IBS-D(379-381). The study also reported increased faecal sulfated BAs in IBS-D patients, which was thought to arise from reduced sulfatase activity in the context of overall reduced BA biotransformation(370). These findings support the presence of dysbiosis and an altered BA pool in IBS-D patients.

Uncontrolled levels of BAs may exert damaging health effects. Increased concentrations of hydrophobic secondary BAs are cytotoxic, resulting in DNA damage and cell death through the induction of oxidative stress and production of reactive oxygen species(382, 383). BAs are important regulators of intestinal homeostasis with antimicrobial and amphipathic properties. At a concentration of 0.5 mM, DCA can successfully prevent bacterial growth in cell culture demonstrating regulation of gut microbial composition through environmental stress(384). Micromolar concentrations of DCA and CDCA may increase intestinal paracellular permeability through phosphorylation of the epithelial growth factor receptor (EGFR), resulting in occludin dephosphorylation and cytoskeletal rearrangement at the tight

junction level(385). In rodent jejunum and ileum, taurocholate and glycocholate (BAs) have demonstrated an apoptotic effect through reduced production of ATP via uncoupling of oxidative phosphorylation and increasing the ATP-ase activity of mucosal homogenates(386).

It is unclear whether gut dysbiosis results in modified BA metabolism or vice versa, although a bidirectional interaction is likely(338).

#### **1.4.4 Dysbiosis in IBS and BAD**

The pathophysiology of IBS remains incompletely understood but may involve an altered gut microbiome. The existence of abnormal colonic fermentation (increased hydrogen colonic gas production seen in IBS patients compared to controls), improvement with antibiotic therapy in 48% of patients with both small intestinal bacterial overgrowth and IBS and a high incidence of IBS after gastrointestinal infections implies a role for gut microbes in IBS as acute enteritis is associated with an increase in mucosal cytotoxic T lymphocytes and an increase in enteroendocrine hypersensitivity with these physiological effects impacting on the gut microbiota environment(129, 228, 387, 388). Increased levels of serum antibodies specific for bacterial flagellins have been observed in patients with post-infectious IBS(389). This reflects the increased abundance of flagellin-producing bacterial species, belonging to *Clostridium* cluster XIVa, which has been demonstrated in IBS(390). Further support of potential association of dysbiosis in IBS is suggested by treatment with probiotic therapy using *Lactobacillus plantarum* 299V, or the VSL3 capsule (mixture of *Lactobacilli* and *bifidobacteria*)(225, 391, 392). These treatments have demonstrated an improvement in IBS symptoms (though not sustained), in particular abdominal pain and bloating which emphasizes the known ability of probiotics in balancing intestinal microbiota.

Evidence of an immune engagement between the gut microbiota and host in IBS has been shown by the increased expression of Toll-like receptors (TLRs) 4 and 5, a family of pathogen-recognition receptors of the innate immune system, in IBS patients, generating a low-grade inflammatory response(393). Mucosa-associated bacteria interact with the intestinal

lumen via TLRs and NOD2 protein. TLRs are located on the processes of dendritic cells that pass through tight enterocyte junctions from the lamina propria into the lumen, as well as being expressed on the apical and basolateral membranes of enterocytes. The pathogenicity of the bacteria determines whether dendritic cells respond aggressively or tolerance is auto-induced through the secretion of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ (312). In addition, significantly elevated levels of human beta-defensin-2 (an antimicrobial protein whose expression is induced by pro-inflammatory cytokines and probiotic microorganisms) were characterized in patients with active IBS compared to healthy controls, signifying an activation of the mucosal innate defense system towards a pro-inflammatory response(394).

Both an increase and decrease in the variation of the gut microbiota has been demonstrated in IBS, as well as the suggestion that the microbiota in these individuals is more heterogenous than that of healthy controls(395). The composition of the gut microbiota has been found to reflect symptom severity in IBS with the presence of the *Ruminococcus torques* phylotype being associated with an increase in severity of bowel symptoms. *R torques* is a recognized mucin degrader and produces pro-inflammatory flagellin proteins therefore this fact may contribute to an altered mucus barrier function(390, 396). An increased abundance of *Cyanobacterium* in IBS subjects has been associated with bloating, satiety and an increased total gastro-intestinal symptom rating scale-IBS score (GSCR-IBS). Abundance of *Proteobacteria* was associated with an increased pain threshold and an increased mental component (on completion of short form-36). The family *Actinomycetacea* has been inversely associated with clinically significant depression in IBS subjects(292). The *Doreo* spp bacteria are the main gas-producing bacteria in the gastrointestinal tract and have been observed to be abundant in patients with IBS. The overproduction of gas is associated with IBS, which may account for the symptoms of abdominal pain and flatulence experienced by these patients(397, 398). Excessive production of gas may result in faster colonic transit time in patients with IBS-D, given that the colons in these patients are more sensitive to increased intestinal



volume than healthy controls(399).

There appears to be a difference in the microbial composition within varying IBS subtypes. *Lactobacillus* and *Collinsella* have been found to distinguish healthy control samples from all IBS subtypes. *Bacteroides* and *Allisonella* characterized IBS-M in particular, IBS-C was composed mainly of *Ruminococci*, while IBS-D contained numerous *Streptococcal* sequences. *Bifidobacterium* clones were minimally present in IBS-D samples(400). Another study investigating the mucosa-associated microbiota in IBS observed a reduction in *Bifidobacteria* in IBS-D compared to patients with IBS-C and healthy controls. IBS-D patients also demonstrated an increase in *Bacteroides* and *Clostridia*(401).

Published data on the microbial composition in IBS subjects are inconsistent and sometimes contradictory. This is likely secondary to high inter-subject variations (phenotypic heterogeneity), differences in molecular techniques employed, environmental factors (e.g. diet, medications) and time point of sampling during fluctuating symptoms (periods of remission, relapse and changes in bowel function) as studies suggest IBS gut microbiomes are less stable(402, 403). Generally, data has revealed an increase in the ratio of Firmicutes to Bacteroidetes in IBS subjects(292, 296, 395, 397, 400, 404). IBS-D patients have been observed to be abundant in streptococci and demonstrate a distinctive set of dominant *Clostridia*(395).

Table 6 summarizes findings of studies of the intestinal microbiome in adult IBS-D patients.

**Table 6: Intestinal microbial composition in IBS-D**

Study	Sample	Method	Findings
<i>Krogius-Kurikka L et al, 2009(404)</i>	Faecal	GC-based profiling and fractioning, 16s rRNA sequencing	↑Proteobacteria, Firmicutes with <i>Lachnospiraceae</i> ↓Actinobacteria, Bacteroidetes
<i>Lyra R et al, 2009(405)</i>	Faecal	16s rRNA sequencing	94% similarity phylotype to <i>Ruminococcus torques</i>
<i>Carroll IM et al, 2011(406)</i>	Faecal	TRFP-fingerprinting of 16s rRNA - PCR	↓Aerobes ↑ <i>Lactobacillus</i>
<i>Maukonen J et al, 2006(407)</i>	Faecal	DGGE, TRAC (hybridization-based technique)	<i>Clostridium coccooides-Eubacterium rectale</i> : 50% of total bacteria
<i>Kerckhoffs AP et al, 2009(408)</i>	Mucosal	FISH, PCR	↓ <i>Bifidobacteria</i>
<i>Tana C et al, 2010(296)</i>	Faecal	PCR, culture	↑ <i>Veillonella, Lactobacillus</i>
<i>Matto J et al, 2005(409)</i>	Faecal	PCR-DGGE, culture-based techniques	↑Aerobe:anaerobe ratio ↑coliforms
<i>Rajilić-Stojanović M et al, 2011(397)</i>	Faecal	Pylogenetic microarray, qRT-PCR	2-fold ↑Firmicutes:Bacteroidetes ratio ↑ <i>Dorea, Ruminococcus, Clostridium</i> ↓ <i>Bifidobacterium, Faecalibacterium</i>
<i>Dior M et al, 2016(370)</i>	Faecal	Real-time PCR	↑ <i>Escherichia coli</i>

## **1.5 16s rRNA gene as a Marker for Profiling of Bacterial Community**

### **1.5.1 Ribosomes and the Bacterial 16s RNA Gene**

In living organisms, the genetic information is stored in the genomic sequences of their DNA (deoxyribonucleic acid). These sequences encode proteins, which are responsible for functioning tasks in living systems. DNA polymerase replicates the genome, to preserve the genetic information in DNA. Consequently, at every cell division, each daughter cell can receive one genome copy. RNA (ribonucleic acid) polymerase undertakes transcription of DNA into mRNA, and translation of mRNA is carried out by ribosomes. Each sequence of mRNA is composed of ribonucleotides, with one of four bases: A (adenine), C (cytosine), G (guanine) and U (uracil). One or several triplets of bases (codons) encode an amino acid. The mRNA sequence is decoded, starting with the AUG codon that is then succeeded by a sequence of codons to specify the order of insertion of amino acids in the emerging protein. This is then followed by a termination codon, indicating that the protein is ready to dissociate from the ribosome to fold into its functional state(410).

Transfer RNA (tRNA) helps to decode the mRNA sequence into a protein and functions at specific sites in the ribosome during translation(410). The loop end of tRNA interacts with the complementary codon of the mRNA that carries the genetic information and is identified as the 'anticodon loop'. The other, single-stranded end, terminates with the CCA nucleotides, and must be in as close proximity to the ribosome as possible, to allow transfer of the peptide bond, at a site known as the peptidyltransferase centre(411).

An intricate mesh of 3 RNAs (23S and 5S for the large subunit; 16S for the small subunit), along with approximately 50 different proteins, forms the bacterial ribosome(411). The rRNA genes are transcribed from the ribosomal operon as 30S rRNA precursor molecules in bacteria and are

then cleaved by RNase III into 5S, 23S and 16S rRNA molecules(412). The helix of the 16S RNA runs vertically along the entire length of the small subunit body, on the interface side, and is referred to as helix 44. It is connected to the decoding center and connects with several bridges in contact with the large subunit, therefore it is thought that helix 44 may have a role in coordinating the actions of the 2 subunits and initiation of translation(411, 413).

In the late 1970s, Carl Woese and George Fox started to analyse and sequence the 16S rRNA genes of various bacteria and discovered the third kingdom, the Archaea, based on phylogenetic taxonomy of 16S rRNA. They demonstrated that phylogenetic trees could be identified by comparing relatively stable parts of the genome (e.g. the 16S rRNA gene)(414). 16S rRNA, which has approximately 1500 base pairs, is the most conserved of the 3 rRNA genes and has been coined as an 'evolutionary clock', which has resulted in the reconstruction of the tree of life(412, 415). Consequently, classification of uncultivable bacteria has been achievable, several bacterial genera and species have been reclassified and the discovery and classification of novel bacterial species has been facilitated(416). Studies have reported 16S yielded genus identification rates of >90%, with species rates of 65-83% and 1-14% of isolates remaining unidentified after testing(417). 16S rRNA is present in all prokaryotic cells and is well adapted for the amplification and measurement of both close and distant phylogenetic relationships due to its conserved and variable sequence regions evolving at very different rates. This enables the use of the 16S rRNA in providing genus and species identification for isolates that do not fit any recognised biochemical profiles(418, 419).

For a large number of bacterial strains, there are dedicated 16S databases, including BLAST (<http://www.ncbi.nlm.nih.gov>) and Seqmatch (<http://rdp.cme.msu.edu>), which include near full-length sequences, enabling sequences from unknown strains to be compared against these sequences(412, 415). In a variety of bacteria, broad-range PCR primers recognise conserved sequences, while amplifying highly variable regions

between the primer binding sites. These regions demonstrate sufficient interspecies variability. The amplified segment is sequenced and then compared with the dedicated databases to identify isolates(420). A new species is demonstrated when 16S rRNA gene sequence data on an individual strain exhibits a similarity score of <97% with a nearest neighbour. However, the definition of similarity scores >97% is not clear as this may represent a new species or signify clustering within a previously defined taxon. Difficulties observed in identifying bacteria include species sharing similar and/or identical 16S rRNA sequences or too few sequences deposited in nucleotide databases(417). Relying on non-full length 16S rRNA gene sequences limits the taxonomic resolution and taxonomic coverage is dictated by the specific hypervariable region(421, 422).

### **1.5.2 Culture-Based and Culture-Independent Approaches to characterizing Bacterial Populations**

In 1881, Robert Koch invented plating techniques and culture-based techniques dominated microbiology for a century. Stains such as Gram, which utilized biochemical or physiological properties, identified microbial species. This method however, is biased and is limited the breadth of detectable bacteria to those that were favoured to proliferate in laboratory culture conditions, such as easily growing, aerobic bacteria like *Escherichia Coli*. In the 1980s, the advent of DNA-based culture-independent techniques arose, changing the microbial landscape and allowing for the investigation of several aspects of microbial communities, including taxonomic composition and functional metagenomics(414). The earliest DNA-based techniques include amplifying specific genes by polymerase chain reaction (PCR), cloning in *Escherichia Coli* and then sequencing(423). Alternatively, fluorescent in situ hybridization (FISH) was utilized, where fluorescently labelled, specific oligonucleotide probes for marker genes were hybridized to the DNA. Since the mid-1970s, DNA sequencing techniques such as Sanger sequencing have been available but this method was observed to be too time consuming for extensive use and expensive(424).

For at least the last three decades, microbial profiling has been centered around the 16S rRNA gene. Environmental metagenomic research provided the basic resources and preceded application to the human body, when in 1990, clone libraries of 16S rRNA genes from environmental bacteria (Sargasso Sea picoplankton) were amplified and sequenced using the Sanger technique(425, 426). In 2005, the revolutionary technology of high-throughput (HTS) or next-generation sequencing (NGS) was announced, which was proven to advance the Sanger method by being more cost and time efficient, as bacterial genomes could be analysed in hours to days rather than months to years(427). Emanating from this was the introduction of two centrally controlled, large-dimensioned research programs – the Metagenomics of the Human Intestinal Tract (MetaHIT) project and the Human Microbiome Project (HMP). MetaHIT aimed to sequence the microbial genomes from faecal samples from both healthy and diseased individuals, while HMP characterized the diversity of the microbiota sampled at multiple body sites in healthy subjects(428, 429).

Despite molecular approaches, including whole metagenome shotgun (WGS) sequencing and 16S rDNA amplicon sequencing, being able to provide in-depth insights into microbial composition, they appear to neglect the detection of low-abundant organisms. A study reported that compared to transmission electron microscopy and Gram stain, 16S rDNA sequencing underestimated counts of Gram-negative prokaryotes(414, 430). However, it is estimated that fewer than 20% of environmental bacteria from all branches of the phylogenetic tree have been discovered and are able to be grown in defined growth media(423). Reasons for this include bacteria failing to grow in conventional media due to inappropriate conditions including pH, temperature and acquisition of essential nutrients. In addition, high-abundant and fast-growing microorganisms may outcompete low-abundant and slower growing ones(414).

In recent times, NGS technologies have developed rapidly with historical methods, including terminal restriction fragment length polymorphism (T-

RFLP), automated ribosomal internal transcribed spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE) being made redundant in favour of high-throughput NGS methods(414). These methods are performed on a single-molecule basis with no initial DNA amplification step(431). At the genomic level, there are two options for NGS-based sequencing of microorganisms. The first, is PCR amplification of the 16S rRNA or other phylogenetically conserved marker sequences with consequent NGS of the constructed amplicon library. Secondly, is whole metagenomic shotgun (WMS) sequencing of the whole genetic content. These techniques include extracting the genomic DNA, constructing appropriate sequencing libraries, NGS, bioinformatic analysis and comparison to dedicated reference databases(414). There are nine variable regions (V1-V9) in the 16S RNA gene, which are discriminating sites and these are essential for accurate richness assessments of microbial diversity(432).

An attractive use of 16S rRNA gene NGS is the identification of rare bacteria or isolates with atypical phenotypic characteristics. The Microseq 500 16S rRNA-based identification system was found to identify 89% unusual aerobic Gram-negative bacilli to the species level and 81% of bacteria with ambiguous biochemical profiles that are clinically significant(420, 433). In general, 16S rRNA sequencing identifies a higher percentage of species than conventional methods. Identification of slow-growing bacteria is an additional advantage of 16S rRNA sequencing whilst reducing the time required to identify these bacteria, such as mycobacteria, which can otherwise take 6-8 weeks to grow in culture. A clinically important use of 16S rRNA sequencing is the identification of uncultivable bacteria, which is achieved through the universal presence and sequence conservation of the 16S rRNA gene, permitting the design of broad-range PCR primers(416).

In order to detect subtle differences between samples or resolve rare species, the sequencing depth of the quantity of reads produced during sequencing is important. Each sequencing technology has a maximum

capacity of number of reads. The quantity of samples that can be analysed simultaneously, as well as the robustness of the data, is influenced by the number of sequence reads that can be undertaken. High depth of coverage permits the detection of more operational taxonomic units (OTUs) for amplicon sequencing-based techniques and for the detection of more genes for WMS analyses(431).

There are several different NGS techniques available on the market with their own platform-specific error profiles. The accumulation of errors may produce false positive variants, overestimating species richness(414). Some have superior read length, accuracy or speed while other sequencing platforms may generate the largest total throughput per run(431). Table 7 demonstrates a comparison of some of the NGS techniques.



**Table 7: Comparison between NGS technologies**

<b>Sequencing platform</b>	<b>454 GS FLX + (Roche)</b>	<b>MiSeq (Illumina)</b>	<b>5500xl W SOLiD (Life Technologies)</b>	<b>Ion PGM (Life Technologies)</b>
<b>Sequencing methodology</b>	Pyrosequencing	Reversible terminator	Ligation	Proton detection
<b>Read length (base pairs)</b>	700	2 x 250	1 x 75 Frag, 2 x 50 MP	100 or 200
<b>Run time</b>	23 hours	27 hours	8 days	3 hours
<b>Reads per run</b>	~1,000,000 shotgun ~700,000 amplicon	6.8 million	1.4 billion x 2	2-5.5 million
<b>Advantages</b>	Fast, read length	High throughput	Accuracy, low cost	Fast
<b>Disadvantages</b>	High cost, low throughput, error rate with polybase more than 6	Short read assembly, high cost	Short read assembly, slower than other methods	Polybase errors

(Adapted from Di Bella and Liu(431, 434)).

## 1.6 Volatile Organic Compounds (VOCs)

### 1.6.1 VOCs in Health and Disease

VOCs are a diverse group of carbon-based chemicals, which are defined by their boiling point (ranging from 50°C to 260°C) and retention time(435). Humans emit a wide selection of VOCs from the body, which are both odorous and non-odorous(436). VOCs exist in the gaseous phase and may be retrieved from the headspace of cells *in vitro*, as well as being present in bodily fluids such as sweat, blood, urine and faeces and emitted from the body in exhaled breath(435, 437). It is thought that VOCs are shared by individuals in health with specific changes occurring in disease. As early as 400 BC, Hippocrates identified the diagnostic value of body odours and reported on various disease-specific odours emanating from sputum or urine(436). Varying profiles of VOCs have been associated with differing diseases including diabetes mellitus, IBS, IBD, colorectal cancer, COPD and TB and therefore display a good relationship with the exposure or disease in question(435, 438). As a result, this and the fact VOCs can be sampled non-invasively and analysed by readily available and simple analytic techniques, renders them as attractive disease biomarkers. This is particularly true given that within a shorter timeframe, VOCs can accurately identify bacterial species compared to traditional culturing methods(439). In their role as biomarkers, VOCs have also been found to be highly sensitive and specific, fast and accurate, require simplistic interpretation and have been thoroughly validated(440).

A compendium of all the VOCs emanating from the human body demonstrates the presence of 1840 VOCs: 381 were identified in the faeces, 279 in urine, 872 in breath, 532 in skin secretions, 256 in milk, 154 in blood and 359 in saliva. Only 12 of these VOCs were found to be omnipresent in all bodily fluids and breath and these included acetaldehyde, 2-propanone (acetone), benzaldehyde, 1-butanol, 2-butanone, hexanal, heptanal, octanal, pentanol, benzene, styrene and toluene(441). The latter three compounds are smoke-derived substances as well as being common environmental pollutants(442). Hydrocarbons represented the greatest

number of VOCs from bodily fluids, which is thought to result from ingestion of a large amount of unsaturated fatty acids in the diet that undergo peroxidation and chain cleavage, producing hydrocarbons such as heptane, pentane, octane and alkenes. The abundance of nitrogen containing compounds was found to be greatest in breath and least in blood. Most sulphur containing compounds were observed in urine, with the least in blood. Out of the VOCs, 225 volatile alcohols and 103 aldehydes were identified with the greatest abundance being present in skin secretions and the least in milk. In total, 102 volatile acids were identified. VOCs were present in certain bodily fluids and not others and this was thought to result from biotransformations by various organs. For example, VOCs present in faeces may not appear in breath due to conversion in the bloodstream by the liver, which may result in some compounds dropping in concentration below the detectable level. A compound detected in blood and not urine may have been transformed by the kidneys(441).

#### *VOCs in Gastrointestinal Disease*

The potential of VOCs playing a role in the early diagnosis of gastrointestinal disease is illustrated by data demonstrating faecal VOCs in neonates who developed necrotizing enterocolitis (NEC) having fewer esters compared to their healthy counterparts. These esters were then reported to have often 'disappeared' from faeces, after being present a few days earlier. This change arose prior to NEC being clinically recognised by physicians(443). Table 8 summarises some of the studies, which have identified key volatiles in gastrointestinal disease.

**Table 8: VOCs in gastrointestinal disease**

Disease	Study	Sample source	Volatile compound
<b>BAD</b>	Covington et al, Sensors, 2013(54)	Urine	↑ 2-propanol, acetamide
<b>IBD</b>	Rieder et al, Clin Transl Gastroenterol, 2016(444)	Breath	↑ 1-octene, 3-methylhexane, 1-decene ↓ 1-nonene, 2-nonene, hydrogen sulfide
	Ahmed I et al, Aliment Pharmacol Ther, 2016(445)	Faecal	↑ Heptanal, 1-octen-3-ol, 2-piperidinone, 6-methyl-2-heptanone ↓ methanethiol, 3-methyl-phenol
	Hicks LC et al, J Crohns Colitis, 2015(446)	Breath	↑ dimethyl sulphide, hydrogen sulphide, hydrogen cyanide, ammonia, butanal, nonanal
<b>IBS-D</b>	Ahmed I et al, PLoS One, 2013(447)	Faecal	↑ cyclohexanecarboxylic acid
<b>Coeliac disease</b>	Arasaradnam RP et al, PLoS One, 2014(448)	Urine	↑ 1,3,5,7 cyclooctatetraene
<b>Hepatic encephalopathy</b>	Goldberg EM et al, J Chromatogr, 1981(449)	Blood	↑ 3-Methylbutanol
<b>Liver Cirrhosis</b>  <b>Advanced fibrosis in chronic liver disease</b>	Fernández Del Río R et al, EBioMedicine, 2015(450)	Breath	↑ Limonene, methanol, 2-pentanone
	Alkhouri N et al, Clin Transl Gastroenterol, 2015(451)	Breath	↑ Isoprene

Table summarizing key volatiles in gastrointestinal disease.

Fermentation of nonstarch polysaccharides by gut microbiota produces an odorous gas composed of various VOCs(238). This is illustrated by a study demonstrating the ability of dogs in correctly identifying the 'smell' of stool samples infected with *Clostridium difficile*(452). Analysis of faecal VOCs from neonates observed fewer VOCs being present in premature neonates compared to healthy adults, with a very low frequency of nitrogen compounds, and barely any sulphides detected at all in the neonatal faeces(453). This reveals the simplicity of neonatal gut microbiota compared to that found in adults as most VOCs are produced by fermentation of dietary substrates by the indigenous gut flora. Volatiles such as ammonia and methanethiol are presumed to derive from methionine by *Clostridium sporogenes* and methanethiol is known to be damaging to colonic epithelium(454, 455).

#### *Mechanisms of VOC Profiles in Disease*

The underlying mechanism of the VOC profiles observed in disease states remains unclear. Quorum sensing, where bacterial cells communicate with each other via diffusible molecules such as VOCs, is regarded as a possible mechanism in regulating bacterial balance(238, 456). It is hypothesized that VOCs reflect microbial metabolic activity and are a surrogate marker for intestinal dysbiosis in disease(360). The resultant VOC profile is evident in urine secondary to altered gut permeability observed in disease(438). As a consequence of disease, pathological processes may either produce new VOCs that are not produced under normal physiological circumstances or that there is an alteration in the concentration of the individual VOCs present(435). There is data that shows a reduced range of VOCs in disease, in particular patients with ulcerative colitis, *Campylobacter jejuni* and *Clostridium difficile*. This study hypothesized that this was secondary to a relatively lower abundance of other intestinal microbes given the abundance of *Campylobacter jejuni* and *Clostridium difficile* in the gut. This diminished biodiversity in bacteria was thought to account for the reduced number of secondary metabolites. It was also thought that the increased transit time observed in these patients with diarrhoeal conditions meant that fewer compounds would be synthesized(457).

In inflammatory processes, VOCs are often compounds manufactured during processes induced by excessive reactive oxygen species (ROS) production such as lipid peroxidation(440). Inflammation has been associated with nitric oxide, nitrate, nitrogen- and sulphur- containing VOCs(458). Volatiles generated during lipid peroxidation include pentane, ethane, hexanal, octanal, nonanal, propanol and butanol(459). Oxidative stress is associated with the pathophysiology of IBD and arises from either an overproduction of ROS or an impairment of the endogenous antioxidant defense system(281). VOCs found in infection are not only derived from the bacteria but are also often related to metabolic processes occurring in the infectious organism as all organisms produce VOCs in their role of metabolism(460). Certain infections bear a distinct smell *in vivo* and *in vitro*(440).

### **1.6.2 Urinary VOCs**

From a chemical perspective, urine is a complex medium, owing to its high number of constituents(461). Its composition is significantly variable and depends on various factors including age, gender, hormonal status, physical activity, food habits and presence of specific pathologies(462-465).

In our experience, urine is the most ‘user-friendly’ of biological samples for patients and clinicians alike in analysing VOCs, compared to faeces, breath and blood. Although breath analysis is another attractive non-invasive option, it is hindered by the difficulty in detecting and quantifying very small amounts of gases/VOCs in the presence of atmospheric gases, which frequently exist in larger molecular weights and quantities. In addition, inhaled particles in breath arise from varying sources and compete with metabolites from oral microbes. Inhaled VOCs may also be degraded endogenously, modifying the ratio between inhaled and exhaled gas concentrations(466).

The compounds in urine are intermediate or end products of various

metabolic pathways, with some of the substances, including alcohol, ketone, pyrrole, furan and sulphide, producing characteristic urine odours(436). Urinary VOC profiles are not only affected by disease, but also by ingestion of foods, with asparagus giving rise to a sulphurous odour(467).

Urinary volatiles encompass a range of molecular classes including alcohols, acids, ketones, aldehydes, amines, N-heterocycles, O-heterocycles, sulphur compounds, hydrocarbons with an insignificant number of esters being described. A significant number of terpenes, which are derived from foods, have been reported(461). A review of urinary VOCs in elderly, generally well men demonstrated five compounds that were present, irrespective of the urine pH - acetone, methylene chloride, 4-heptanone, 2-pentanone and 2-butanone(468). The large quantity of ketones seen in urine is likely to arise from decarboxylation from corresponding oxo-acids by intestinal bacteria(461). During periods of rapid fat oxidation, acetoacetate, hydroxybutyrate and propanone are manufactured in the liver, when the rate of fat breakdown surpasses the capacity of the Krebs cycle to process the ensuing acetylCoA(469).

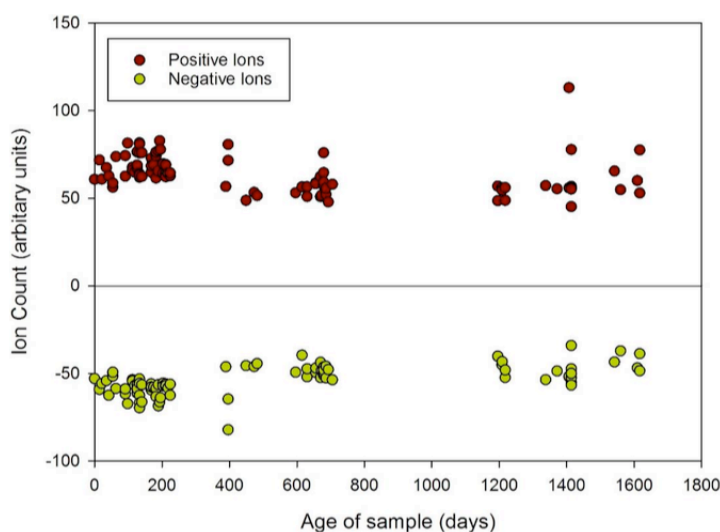
Despite being able to identify specific classes of urinary volatiles, there is a paucity of data quantifying their specific measurements in urine. Varying levels of significant ketone bodies, propanone (acetone) and acetoacetate, at 1.16–14 mmol L<sup>-1</sup> and 1.3–15 mmol L<sup>-1</sup> respectively, have been observed(470). Increasing protein intake has been observed to increase concentrations of p-cresol (typically 52 mg day<sup>-1</sup> excreted in urine) and phenol (typically 10 mg day<sup>-1</sup> excreted in urine). These compounds are thought to originate from intestinal microbial action on tyrosine with aerobic bacteria in the ileum/caecum producing p-cresol and in the left colon, anaerobic bacteria producing phenol. With the addition of a high fibre diet to ingestion of high protein, the resulting decreased transit time causes a smaller increase in these compounds(471).

With regards to urinary volatile short chain fatty acids (and semi volatile acids), the greatest concentrations are as follows: hippuric > glycolic >

benzoic > ethanoic > 2-ketoglutaric > 2- hydroxyisovaleric > lactic, 2-hydroxyisobutyric > oxaloacetic > pyruvic acids, with isobutyric, propionic, butyric and 2- methylbutyric acids in minor concentrations(472).

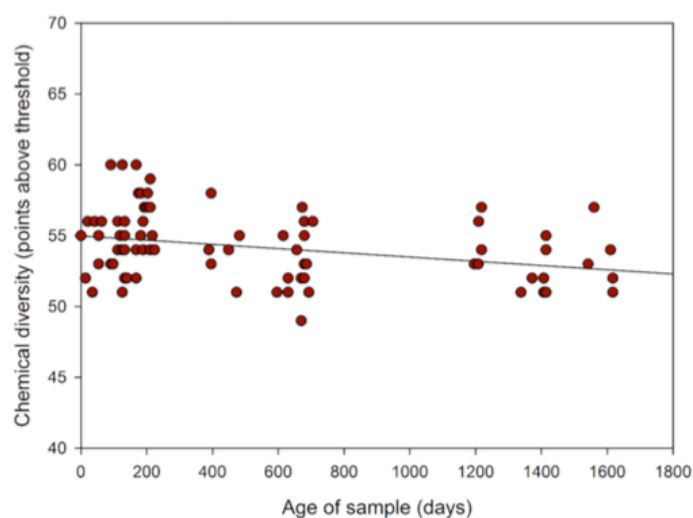
Our research group have demonstrated that total gas/vapour emissions from urine samples reduced over time with loss of chemical signal, as the samples aged. In the initial nine months of storage, there was less variation with greater stability and uniformity of concentrations of VOCs together with tighter clustering of the quantity of chemicals released. As equilibrium is reached between the urine and airspace in the storage container, all vapours and gases will emanate from a sample over time. Potential reasons for loss of chemical signal as urine samples age include water in the urine evaporating over time with the consequent release of water-soluble volatiles, bacterial activity in very old samples causing a higher chemical output and the plastic from the standard specimen storage bottles absorbing volatiles over time. As a result, we proposed that nine months could be considered a general reference to the shelf-life of a urine sample(473). This is demonstrated in the two graphs (figure 3) below showing (a) a change in total number of urinary VOCs over time and (b) chemical diversity of urinary VOCs over time.

**Figure 3a: Change in total number of urinary VOCs over time (from December 2009 to May 2014)(473).**





**Figure 3b: Chemical diversity of urinary VOCs over time (from December 2009 to May 2014), with linear fit to emphasise output change(473).**



### **1.6.3 VOCs in IBS and BAD**

There is a significant lack of data, investigating the use of VOCs as biomarkers in IBS and BAD. To date, there are only three IBS studies and one BAD study, the latter being undertaken by our research group. The studies are outlined in table 9.

**Table 9: VOC studies in IBS and BAD**

<b>Study</b>	<b>Disease</b>	<b>Sample</b>	<b>Analytical Method</b>	<b>VOCs identified</b>
Ahmed I, et al. PLoS One, 2013(447)	IBS-D CD UC HC	Faecal	GC-MS	IBS-D: esters of short chain fatty acids, cyclohexanecarboxylic acid and its ester derivatives .  IBD: aldehydes
Baranska A, et al. Aliment Pharmacol Ther, 2016(474)	IBS HC	Breath	Thermal desorption-gas chromatography combined with time-of-flight mass spectrometry	IBS: n-Hexane; 1,4-Cyclohexadiene, n-Heptane, Aziridine (Ethylenimine)
Arasaradnam RP, et al. PLoS One, 2014(448)	IBS-D Coeliac disease	Urine	FAIMS and GC-MS	Coeliac: 1,3,5,7 Cyclooctatetraene (not found in IBS-D)
Covington JA, et al. Sensors, 2013(54)	BAD UC HC	Urine	AlphaMOS Fox 4000 electronic nose, FAIMS and GC-MS	BAD: 2-propanol and acetamide (much reduced or not present in UC/HC groups)

#### **1.6.4 Analytical Techniques used in the Study of VOCs**

Only recently, has there been an explosion in an interest in the analysis of VOCs for medical purposes. Analyses of VOCs have already been established and are routinely used in the assessment of forensic science, cosmetics, environmental contamination and the fragrance and flavor industries(475).

A single analytical method may not be suitable for measuring VOCs from various bodily fluids and breath and it is usual practice for researchers to utilize equipment that is available to them. Not all reported VOCs may be endogenous in origin and some may arise from artifacts including oxidation or degradation and contamination, which may result during the collection, storage or measurement of samples(441).

##### *GCMS*

There are several analytic methods, which may be used to detect VOCs with gas chromatography mass spectrometry (GCMS) being the main technique employed. Compound identification of VOCs using GCMS may be inaccurate as spectral library matches on their own are often used to identify peaks, which may be misleading for isomers. The compound may not also be present in the MS libraries or there may be mass spectral similarities, predominantly with hydrocarbons and isomers, resulting in misidentification of the VOC(441). More recently, the addition of retention time matching to library matching has been applied and this 'double check' allows for increased accuracy in identifying VOCs(441). In direct mass spectrometric methodologies, the recorded spectrum reflects the composition of the total sample, as there is no separation of sample components prior to ionization. This may cause some ambiguity, as the fragmentation pattern of ions of particular mass-to-charge ratio needs to be considered when being assigned to certain substances(441).

##### *FAIMS*

Field asymmetric ion mobility spectroscopy (FAIMS) is an instrument, which separates ionised gas molecules at room temperature and atmospheric

pressure. Separation of gas/vapour molecules in the ionised samples produces an asymmetric waveform. This is achieved by the sample being inserted between two metal plates, where an asynchronous high-voltage waveform has been applied, causing some ions to remain between the plates and others to drift and hit the plates. A complex composition of gases can be separated by their differences in mobility in high electric fields by applying a range of dispersion and compensation voltages(456).

### *Electronic Nose*

The electronic nose is a term used to identify a method rather than specific analytical technology. These instruments emulate the human olfactory system in identifying patterns in a collection of non-specific sensors, rather than detecting individual chemical components. An array of 8-32 differing chemical sensors form a conventional electronic nose, which broadly detect various chemical groups including ketones, alcohols and low pressure gases(466). An electronic nose generally consists of three different systems – sample delivery, detection and data computing(465). The ‘headspace’ (air above the biological sample) is injected into the electronic nose, resulting in a unique response from each sensor within the array(466). The profile of sensor responses is used to produce a ‘fingerprint’ of an aroma(456). The most commonly used sensors are polymeric, metal-oxide and quartz microbalance sensors(465). It is possible to teach the device to recognise a variety of different conditions by extracting a feature of the sensor response (e.g. the maximum change) and employing a pattern recognition engine. Therefore, when the device is delivered a sample from the same disease group, the sensors are able to repeat the response pattern, allowing for identification of the disease(465, 466). Multivariate statistical analysis is used to process the data, the most common being linear discriminant analysis (LDA) and principal component analysis (PCA). LDA aims to accomplish maximum separation of data groups and dimensional reduction before future classification by presenting an algorithm, which identifies a linear combination of features characterizing or separating two or more events or objects. PCA maintains the main information present in the

original data by aiming to visualize the data within a low dimensional space(465).

Table 10 describes the advantages and disadvantages of some of the modalities used to analyse VOCs in clinical practice.

**Table 10: Analytical techniques in VOC studies**

<b>Technique</b>	<b>Electronic nose</b>	<b>GCMS</b>	<b>FAIMS</b>
<b>Breadth of analysis</b>	Medium	High	High
<b>Sensitivity</b>	High	Very high (pre-concentration required)	High
<b>Specificity</b>	Medium	Very high	High
<b>Accuracy</b>	High	High	High
<b>Speed</b>	Real-time	Off-line	Real-time
<b>User skill level</b>	Low	High	High
<b>Consumable cost per item</b>	Low	Medium	Low
<b>Maintenance</b>	Low	High	Medium
<b>Sample cost</b>	Low	Medium	Low
<b>Estimated cost (£)</b>	<40,000	>150,000	<50,000

(Adapted from Arasaradnam RP et al, Review article: next generation diagnostic modalities in gastroenterology - gas phase volatile compound biomarker detection. Aliment Pharmacol Ther. 2014)(466)

# **Chapter 2: The Gut Microbiome in BAD and IBS-D**

## 2.1 Introduction and Aims

### 2.1.1 Introduction

The human intestinal microbiota is responsible in regulating gut homeostasis and has become the focus of extensive interest in recent years due to its implied role in precipitating host disease. The complexity of this massive and diverse community, harbouring approximately  $10^{14}$  microorganisms of 500 to 1000 bacterial species, remains poorly understood(18).

The gut microbiota maintains intestinal homeostasis, both directly and indirectly via bile acid receptors and bile acid signalling. Primary bile acids (cholic acid – CA; chenodeoxycholic acid - CDCA) undergo biotransformation through a series of reactions by the gut microbiota to form secondary bile acids (deoxychenocholic acid – DCA, lithocholic acid - LCA). These reactions include deconjugation (removal of the amino acid side chain), epimerization (of 3-, 7-, and 12-hydroxy groups), oxidation (removal of H), dehydroxylation (replacement of a hydroxyl group with a hydrogen), and hydroxylation (replacement of a hydrogen with a hydroxyl group)(17). The resulting bile acid metabolites act as signaling molecules via the TGR5 (G protein coupled bile acid receptor) and farnesoid X receptor (FXR) to inhibit inflammation, prevent pathogen invasion and maintain the integrity of the gut epithelial barrier(360).

In bile acid diarrhoea, interruption of the normal enterohepatic recirculation of the recycling of bile acids ensues in an excess of bile acids entering the colon. This is significant as only certain microbial populations, which are able to tolerate normal physiological concentrations of bile acids in the intestine, are able to survive. The amphipathic nature of bile acids which allows them to act as detergents of dietary fats also enables them to interact with bacterial lipid membranes, conferring them with potent antimicrobial abilities in breaching the integrity of the cell membrane, resulting in leakage of cellular components and apoptosis(17, 377). The concept of dysbiosis of

pathological imbalances occurring in gut bacterial colonies has been used to explain the initiation and outcome of disease in the host. Modification of bile acid hormones is influenced primarily by the intestinal microbiota and may perturb regulatory FXR-mediated signalling, resulting in impaired intestinal homeostasis(476).

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder with a prevalence of 10-25% in developed countries and despite its clinical significance, the underlying pathophysiology of this debilitating condition remains ambiguous(158). Bile acid diarrhoea (BAD) is commonly overlooked in the differential diagnosis of chronic diarrhoea and has been demonstrated in excess of a quarter of patients who were previously diagnosed with IBS-D(39).

Although the aetiology of IBS is multifactorial, the impact of the gut microbiota has generated much attention. Changes observed in the faecal microbiota composition, the presence of abnormal colonic fermentation with increased hydrogen colonic gas production, improvement of symptoms with antibiotic therapy, and an increased incidence of IBS after gastrointestinal infections imply a role for gut microbiota in IBS as acute enteritis is associated with an increase in mucosal cytotoxic T lymphocytes and an increase in enteroendocrine hypersensitivity which will impact the gut microbiota environment(129, 387, 477). Production of the pro-inflammatory cytokines including TNF-alpha, IL- 1, and IL-6 causing an augmented cellular immune response as well as significantly elevated levels of human beta-defensin-2 (expression induced by pro-inflammatory cytokines and probiotic microorganisms) detected in patients with IBS further supports the pathological role of gut microbiota(394, 478). The hypothetical association of dysbiosis in IBS and balancing of the gut microbiota is suggested by the improvement in symptoms observed with probiotic therapy using *Lactobacillus plantarum* 299V, or the VSL3 capsule (mixture of *lactobacilli* and *bifidobacteria*)(225, 391, 392). Despite numerous studies profiling the gut microbiome in IBS, no research has been similarly undertaken in patients with BAD.



### **2.1.2 Aims**

This study is the first to investigate the faecal microbiome in patients with BAD. The aim of this study was to compare the faecal bacterial composition in patients with IBS and BAD using 16S rRNA gene sequencing.

## **2.2 Materials and Methods**

### **2.2.1 Recruitment and Sample Collection**

#### ***Ethical Approval***

Patients were recruited as part of the FAMISHED (Food and Fermentation using Metagenomics in Health and Disease) study. Scientific and ethical approval was acquired from the local Research and Development Office as well as Warwickshire Ethical committee ref: 09/H1211/38. Written informed consent was obtained from all participants in the study.

#### ***Study Participants***

Patients from the Nuclear Medicine department at University Hospitals Coventry and Warwickshire (UHCW NHS Trust) were recruited from April 2012 to May 2015 after being referred for a SeHCAT scan via attending a gastroenterology clinic with chronic diarrhoea. 14 patients with Rome III criteria IBS-D and 20 patients with BAD in total participated in the study.

Demographic and clinical data including age, gender, ethnicity, BMI, C-reactive protein (CRP – marker of inflammation) and faecal calprotectin levels (a measurement of the protein calprotectin in the stool, which is elevated during intestinal inflammation therefore used to ensure patients with IBD are in remission) for patients with type 1 BAD and co-existing IBD, SeHCAT result, severity and type of BAD were collected.

### ***Sample Collection***

Stool samples were collected in standard specimen collection bottles in the nuclear medicine department and stored at -80°C within two hours of collection. They were transferred to the University of Warwick on dry ice (-78°C) within a polybox and then stored at -80°C. The samples were then thawed on ice for 45 minutes prior to DNA extraction.

### ***Inclusion and Exclusion Criteria***

To be included in this cross-sectional study, both male and female participants were required to meet the following criteria: (1) have chronic diarrhoea, (2) diagnosis of BAD based on a SeHCAT retention value of  $\leq 15\%$  (3) patients with IBD (inflammatory bowel disease) may only be included if type 1 BAD is present, (4) patients who have had a previous cholecystectomy may only be included if type 3 BAD is present.

Participants were excluded from the study if they suffered from coeliac disease, IBD (if type 1 BAD is not present), active IBD (defined as FCP >50 mg/kg stool or CRP >11 mg/L at the time of the SeHCAT scan), colorectal cancer or had been on antibiotics/probiotics in the last three months.

## **2.2.2 DNA Extraction, Quantification, PCR and Purification**

### ***(i) Isolation of DNA from stool samples using QIAamp Fast DNA Stool Extraction kit (Qiagen, UK)***

1 mm glass beads were inserted into the bottom of a 2 ml microcentrifuge tube and 200mg of stool was weighed out into the tube. To help the lysis of cells, 0.2g of 100-300  $\mu\text{M}$  acid washed glass beads (Sigma-Aldrich, Poole, UK) were added followed by disruption with 2 x 30 sec pulses at 6.2 m/s in a FastPrep FP120 machine. 1 ml InhibitEX buffer was added to the stool sample and then vortexed for 1 minute until the stool sample was thoroughly homogenized. The purpose of the InhibitEX buffer is to bind potential PCR inhibitors in the sample.

The suspension was heated for 5 minutes at 70°C, vortexed for 15 seconds and then centrifuged for 1 minute at 13,000 rpm to pellet stool particles. 15 µl of Proteinase K was pipetted into a new 1.5 ml microcentrifuge tube. 200µl of the centrifuged sample was added to the tube containing Proteinase K. 200 µl of buffer AL was then added and the tube was vortexed for 15 seconds. Proteinase K degrades and digests proteins in the sample and buffer AL is a lysis buffer.

The tube was incubated at 95°C for 10 minutes, 200 µl of 100% ethanol was added to the lysate and mixed by vortexing. 600 µl of lysate was applied to the QIAamp spin column and then centrifuged at 13,000 rpm for 1 minute. The QIAamp spin column was then placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded. 500 µl of buffer AW1 was added to the QIAamp spin column and then centrifuged at 13,000 rpm for 1 minute. The QIAamp spin column was then placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded. 500 µl of buffer AW2 was added to the QIAamp spin column and then centrifuged at 13,000 rpm for 3 minutes. The QIAamp spin column was then placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded. The QIAamp spin column was then centrifuged at 13,000 rpm for another 3 minutes to remove any residual buffer. AW1 buffer contains an increased proportion of ethanol to improve the pH conditions and remove excess salt. AW2 is a lengthier spin to remove digested proteins or other impurities. The QIAamp spin column was transferred into a new 1.5 ml microcentrifuge tube. 200 µl of Buffer ATE was pipetted directly onto the QIAamp membrane. It was then incubated for 1 minute at room temperature and centrifuged at 13,000 rpm for 1 minute to elute DNA. Eluted DNA was stored at -20 °C until required.

#### ***(ii) DNA Quantification***

The eluted DNA was quantified using the broad-range Qubit kit. A working solution was prepared by diluting Qubit dsDNA reagent (Life technologies, USA) 1:200 with dsDNA buffer. 190 µl of working solution for standards and

198 µl for samples were added to 0.5 ml tubes. 10 µl of Qubit standard was added to the standard tubes and 2 µl of sample was added to the sample tubes. The tubes were mixed by vortexing for three seconds and then incubated at room temperature in the dark for two minutes prior to quantification on the fluorometer and the concentrations recorded.

### ***(iii) Polymerase Chain Reaction (PCR)***

To amplify genes for coding and sequencing, PCR was used. All designed primer pairs were manufactured by Life Technologies and had similar melting temperatures ( $T_m$ ). The primer sequences are outlined in table 11. V3-V4 primers and extensor ready mix (Thermo scientific) were used to amplify the 16s rRNA gene v3-v4 fragment from isolated metagenomic DNA using the following PCR thermal cycler program:

- 95°C for 3 minutes
- 25 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

***Table 11: Primer sequences that were used in this study***

Region	Primer Sequence 5'-3'
V3F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG
V4R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC

### ***(iv) Analysis of DNA by Agarose Gel Electrophoresis***

After PCR, the DNA samples were analysed by electrophoresis on 1% agarose gels (figure 4). Agarose was dissolved in the working stock of TAE buffer and then melted in a microwave. 5 µl of SYBR safe (Life Technologies) was added to 50ml melted agarose once it had cooled

sufficiently. The agarose was then poured into a plastic cast and wells were created by inserting a plastic comb, which was removed once the agarose gel had set. The gel was then inserted into an electrophoresis tank with further TAE buffer added to ensure the level was high enough to cover the gel. Loading dye was added to each sample at a ratio of 5:1 and run at 100 volts for 40 minutes. On all gels, Hyperladder 1 kb (Bioline, UK) was utilized as the DNA ladder. The gel was then transferred to a BioRad gel-doc system to visualize the DNA.

**Figure 4: Gel electrophoresis of the BAD samples** - DNA bands separated on a gel with the length of the DNA fragments being compared to the DNA ladder (marker on far left – Hyperladder 1kb) containing fragments of known lengths.



#### **TAE Buffer**

This buffer was prepared as a 50 x stock solution and diluted to 1 x with distilled water for the working solution. The stock solution was made up of 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) for one litre.

#### **(v) Post-PCR DNA Purification**

After checking for DNA by gel electrophoresis, the samples were then purified. After PCR, 22.5 µl of dH<sub>2</sub>O was added to 22.5 µl of the post-PCR

product. 72 µl of AMPure beads were then added and mixed by pipetting up and down 10 times before incubating for 10 minutes at room temperature. A magnetic rack was used to pellet the beads against the side of the low-binding microfuge tube and the supernatant was then removed. 200 µl of 80% ethanol was then used to wash the beads twice and then the beads were air dried for 10 minutes at room temperature. The beads were resuspended in 20 µl of elution buffer and then placed on the magnetic rack to pellet the beads against the side of the low-binding microfuge tube with the supernatant being removed. Purified DNA was stored at -20°C until required.

***(vi) DNA Sequencing: Illumina 16S rRNA gene sequencing library preparation***

PCR products were diluted to 4nM and pooled in equimolar amounts.

To denature the DNA, 5 µl of the library pool was added to 5 µl of 0.2 N NaOH, which was, then vortexed and centrifuged at 300 rpm for 1 minute. The denatured pool was then incubated for 5 minutes at room temperature before 990 µl of chilled HT1 buffer was added. This was then vortexed and centrifuged at 300 rpm for 30 seconds before 360 µl of the mixture was added to 240 µl of chilled HT1 buffer in a new tube to make up a concentration of 12pM. The denatured DNA pool was then sequenced on a MiSeq using the Illumina Miseq V2 2x300 bp paired end protocol.

**2.2.3 Statistical and Bioinformatic Analysis**

Demographic data are presented as means and standard deviations (SD). Default Illumina software trimmed sequences to remove adapter sequences, primers, barcodes and low-quality reads. Sequences with less than 1000 reads were removed. Using a custom java program, formation of contigs was performed by joining together forward and reverse reads with a quality-filtering step to remove contigs that had more than three mismatches. The contigs were de-replicated and then clustered at 97% identity to form OTUs using the UPARSE pipeline. Singleton contigs from the dataset were discarded and chimeras were removed. The UPARSE pipeline calculated

the abundance of each OTU by mapping the de-replicated contigs against the OTUs sequences. Taxonomy was assigned to 16s RNA gene OTU sequences using QIIME (Quantitative Insights into Microbial Ecology) and the RDP classifier.

Using the QIIME pipeline, the level of alpha diversity in our samples was determined by generation of rarefied OTU tables, computing measures of alpha diversity for each rarefied OTU table, collating the rarefied OTU tables and then generating rarefaction curves. The depth of rarefaction was defined by either the lowest number or median number of sequences assigned to a sample within a group that was analysed. The Shannon index was used to calculate alpha diversity indexes from rarefied samples.

QIIME calculated beta diversity using weighted and unweighted UniFrac. Rarefaction was performed on OTU tables to remove sample heterogeneity.

## 2.3 Results

### Demographic Data

Demographics of the 34 patients are seen in the Table 12.

**Table 12: Demographic data of the study participants**

	<b>BAD (n=20)</b>	<b>IBS (n=14)</b>
<b>Age (years)</b>	56.9 ± 12.4	45.3 ± 15.6
<b>Gender</b>	12/20 (60%) F 8/20 (40%) M	8/14 (57.1%) F 6/14 (42.9%) M
<b>Ethnicity</b>	18/20 (90%) Caucasian 2/20 (10%) Indian	12/14 (85.7%) Caucasian 2/14 (14.3%) Indian
<b>BMI</b>	28.7 ± 6.5	27.8 ± 5.6
<b>SeHCAT value (%)</b>	6.9 ± 0.04	33.6% ± 0.2
<b>Severity of BAD</b>	3/20 (15%) Mild 8/20 (40%) Moderate 9/20 (45%) Severe	N/A
<b>Type of BAD</b>	3/20 (15%) Type 1 14/20 (70%) Type 2 3/20 (15%) Type 3	N/A

- 26 of the 34 patients (15 with BAD and 11 with IBS) had a body mass index (BMI) recorded.
- Results expressed as mean ±SD.

The BAD cohort of patients (12 female and 8 male) aged 28 to 79 years had a mean BMI of 28.7, defining them as being overweight. The mean SeHCAT value was 6.9%, which is consistent with moderate BAD. Of the 20 patients, 3 (15%) had mild, 8 (40%) had moderate and 9 (45%) had severe severity of disease respectively. Most (14 – 70%) patients had type 2 BAD, 3 (15%) patients had type 1 and 3 (15%) had type 3 BAD.



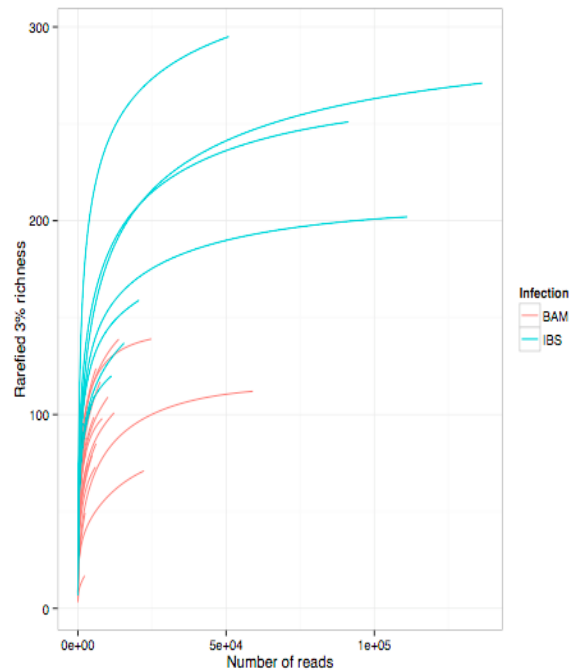
The IBS cohort of patients (8 female and 6 male) aged 20 to 77 years had a mean BMI of 27.8, defining them as being overweight. The mean SeHCAT value was 33.6%, which excluded them from having BAD.

## **OTUs**

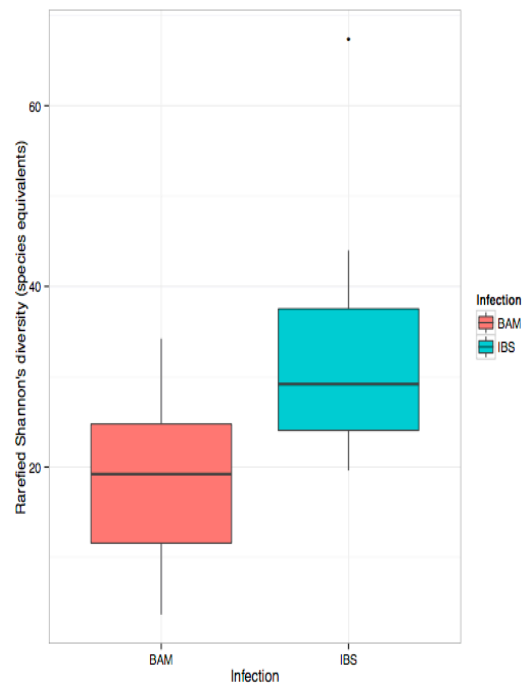
Of the samples collected, 668 OTUs were identified. There was significant difference in the diversity of the OTU in BAD vs IBS patients ( $p = 0.007216$ ). This is demonstrated in the rarefaction curve (Figure 5a) and Shannon's diversity box plot (Figure 5b) seen below, where BAD patients are observed to have reduced diversity compared to IBS patients. The overall variation observed in the bacterial communities in BAD and IBS patients is 8%. The heatmap plot and dendrogram linkages (Figure 6) seen below depicts the relative abundance of each bacterial family in the samples (variables clustering on the Y-axis) within each OTU (X-axis clustering). There were significant differences in the abundances of 10 OTUs, but this was not robust to adjusted  $p$  values. Of these 10 OTUs, 6 OTUs (OTUs 283, 17, 268, 127, 319 and 553) were more abundant in patients with BAD vs IBS and 4 OTUs (OTUs 136, 519, 72 and 356) were more abundant in IBS vs BAD. These are outlined in Table 13 and plotted as box plots in Figure 7, which demonstrate the relative abundance of each OTU in patients with BAD vs IBS.

**Figure 5: (a) Rarefaction curve (b) Shannon's diversity box plot.**

**(a)**



**(b)**

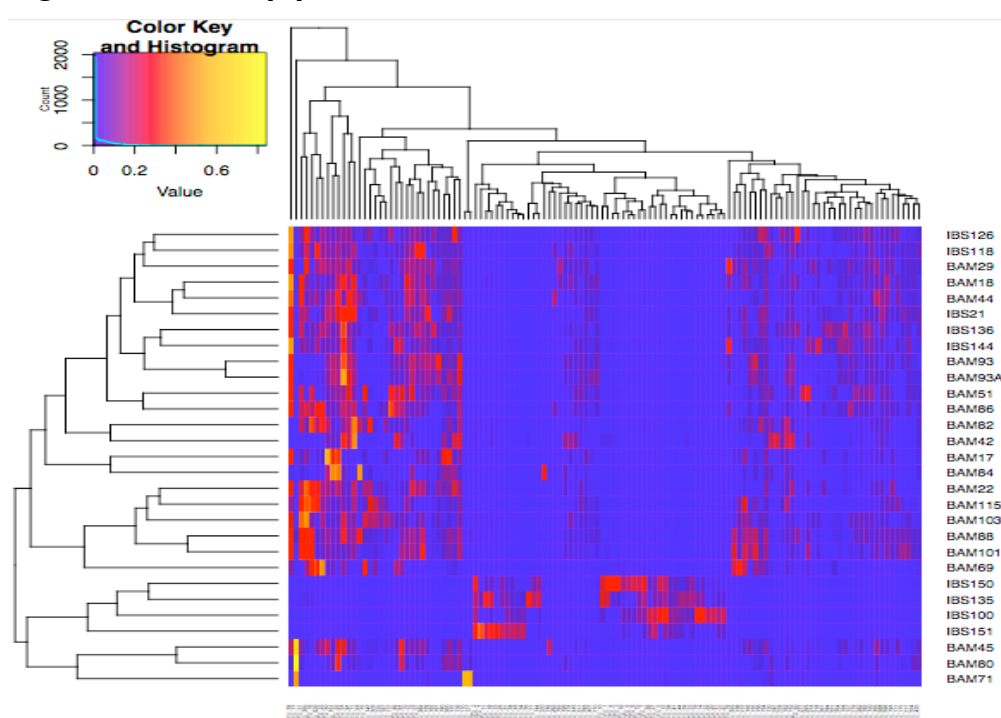


Both demonstrate reduced bacterial diversity in patients with BAD vs IBS.

**(a)** Rarefaction analysis is a calculation of species richness for the limited number of samples analysed. The growth of the curve is rapid at first as the most common species are identified but then plateaus once the rarest species remain to be found.

**(b)** The box plot compares bacterial diversity at the rarefied level.

**Figure 6: Heatmap plot**



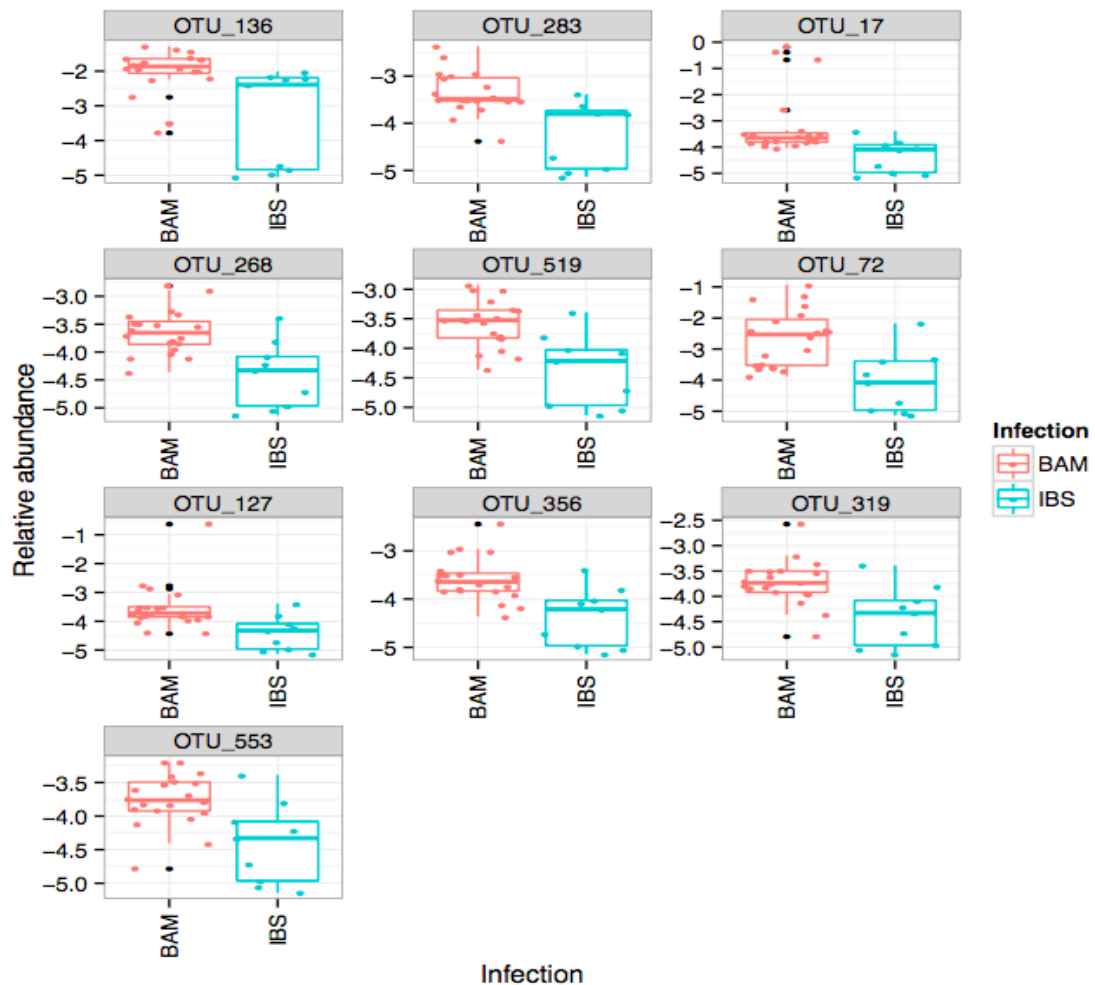
Heatmap plot depicting bacterial diversity and relative abundance of OTUs in the samples collected by colour intensity with the legend indicated at the top left of the figure. For each sample, the plot describes the relative abundance of each taxonomic class and aids in determining which samples have similar phylogenetic profiles.

**Table 13: OTUs whose abundance is significantly higher in BAD (light grey) or IBS (white).**

OTU	BAD_mean	IBS_mean	<i>p</i>	<i>pa</i>	Bacteria (family)
<b>136</b>	1.978952 e-02	4.046352 e-03	0.001348043	0.1986392	<i>Lachnospiracheae</i>
<b>283</b>	6.588814 e-04	4.547205 e-05	0.001348043	0.1986392	<i>Bifidobacteriaceae</i>
<b>17</b>	7.387914 e-02	1.247412 e-05	0.002183045	0.1986392	<i>Prevotellaceae</i>
<b>268</b>	2.048619 e-04	0.000000 e+00	0.002979467	0.1986392	<i>Lachnospiracheae</i>
<b>519</b>	2.375111 e-04	5.384334 e-06	0.003470017	0.1986392	<i>Ruminococcaceae</i>
<b>72</b>	1.504455 e-02	8.195882 e-04	0.004033018	0.1986392	<i>Bacteroidaceae</i>
<b>127</b>	1.620633 e-02	0.000000 e+00	0.004677735	0.1986392	<i>Prevotellaceae</i>
<b>356</b>	3.626509 e-04	5.384334 e-06	0.004677735	0.1986392	<i>Ruminococcaceae</i>
<b>319</b>	1.859629 e-04	0.000000 e+00	0.007209571	0.1986392	<i>Verrucomicrobiaceae</i>
<b>553</b>	7.954283 e-05	0.000000 e+00	0.008293898	0.1986392	<i>Bacteroidaceae</i>

This table demonstrates the mean (BAD and IBS) log values of specific OTUs that were found to have significant differences in abundance in BAD and IBS. *p* values denote significance levels; *pa* values denote corrected significance levels.

**Figure 7: Box plots demonstrating the relative abundance of each OTU in patients with BAD vs IBS-D.**



The box plots demonstrate the median values and interquartile ranges for each OTU.

## 2.4 Discussion

These results demonstrated that there were significant differences in the intestinal milieu of bacterial species in patients with IBS and BAD. Six OTUs were observed to be more abundant in patients with BAD compared to those with IBS. Bacterial diversity was significantly reduced in patients with BAD compared to those with IBS, despite the abundance of various anaerobic taxa, including *Bifidobacteria*, *Prevotella*, *Lachnospiraceae*, *Verrucomicrobia* and *Bacteroides*. The presences of *Prevotella* and *Ruminococcus* taxa have previously been shown to be indigenous members

of a healthy intestinal microbiome, whereas this data contradicts this finding as both taxa were observed in abundance in those with BAD and IBS, respectively(341). The occurrence of reduced bacterial diversity, parallels that of patients with inflammatory bowel disease (IBD), where a loss of normal anaerobic bacteria such as *Bacteroides*, *Lactobacillus* and *Eubacterium* species has been observed(479). Using PCR and culture based techniques, the intestinal microbiome in IBS patients has also demonstrated an increased aerobe to anaerobe ratio with decreased numbers of *Bifidobacteria* and *Lactobacilli*(409, 480, 481). This study data resembles this observation with *Bifidobacteria* being found to be more prevalent in BAD, rather than in IBS.

*Ruminococcus* lies within the Firmicutes phyla and the greater abundance of this genera demonstrated in patients with IBS rather than in BAD contradicts animal data, which demonstrated that rats who were fed a diet containing cholic acid, resulted in an increase in the Firmicutes proportion of the intestinal microbiota from 54% to 95% - specifically, an increase in *Ruminococcus* was noted(377). *Ruminococcus* is a 7 $\alpha$ -dehydroxylating bacteria and is capable of converting CA to DCA, which has greater antimicrobial properties compared to CA(482). Consequently, we hypothesize that an increased proportion of primary bile acids (CA) in the bile acid pool will result in reduced bacterial diversity. Our findings may have occurred due to a shift in the bile acid pool from unexpected reduced amounts of *Ruminococcus*, resulting in a disproportionate increase in cholic acid, which is less bactericidal, allowing for the greater survival and outgrowth of other microbes, which all compete for nutritional resources. Therefore, there would not be a selective enrichment of the Ruminococcaceae bacteria in this environment. This hypothesis is supported by a study conducted in liver cirrhosis, which demonstrated a reduced bile acid pool with decreased conversion of primary to secondary bile acids being associated with decreased intestinal bacterial diversity. With increasing disease severity, a reduction in Ruminococcaceae was also observed with a positive correlation between this taxa and DCA/CA ratio

( $r=0.82$ ). Dysbiosis was evident with an expansion in pathogenic, pro-inflammatory taxa(483).

The dysbiosis observed in this study may have resulted in disease through modified bile acid metabolism - a theory reinforced in IBD patients where intestinal dysbiosis was associated with bile acid dysmetabolism. In this study, a decrease in secondary bile acids and an increase in the sulphated form, which *in vivo*, had the potential to induce the production of pro-inflammatory cytokines by colonic epithelia was demonstrated. Interestingly, despite the reduction in secondary BA concentrations, the overall BA pool size was maintained with normal overall total BA levels. This decrease was thought to arise from impaired intestinal metabolism of BAs by the gut microbiota. This hypothesis was strengthened by the observation of germ-free mice exhibiting undetectable levels of secondary BAs and a higher proportion of conjugated and sulphated BAs, compared to conventional mice, illustrating the role of microbiota enzymatic activity(374). Gut bacteria, including *Bacteroides* (which were found to be abundant in our BAD patients) and *Clostridium*, are known to support sulphatase activity(484, 485), which may have further contributed to the dysbiosis observed in our study. Consequently, colonic epithelial inflammation is enhanced, thereby creating a hostile environment for other groups of bacteria to flourish. This in turn, may result in limiting the presence of bacteria bearing bile salt hydrolase activity (important in the biotransformation of primary to secondary BAs).

As well as 7 $\alpha$ -dehydroxylation reactions increasing the hydrophobicity of bile acid molecules, other reactions by bacterial enzymes contribute to their antimicrobial effects, which may result in the reduction and expansion of certain bacterial communities. Hydrolysis of conjugated BAs by bile salt hydrolases (BSHs) yields free bile acids. Conjugation of BAs demonstrates strong bactericidal activity and bacterial BSH production appears to aid detoxification of bacterial microenvironments(486). BAs have demonstrated various activities on bacterial cells including interrupting macromolecule

stability, inducing DNA damage and activating enzymes involved in DNA repair(487). These BSHs are evident in *Bifidobacterium* and *Bacteroides*, both of which were detected in greater abundance in patients with BAD(18). Through dysbiosis, the greater BSH activity and reduced dehydroxylation effects (as increased *Ruminococcus* was observed in our IBS cohort) that may occur in our BAD patients, may represent limiting steps in the bile acid biotransformation pathway for an effective bile acid pool as further bacterial enzymatic reactions including dehydroxylation and dehydrogenation can only occur after deconjugation(486). This may potentially trigger disease as modification of bile acid composition influences host health and metabolism(486).

Intestinal bacterial hydroxysteroid dehydrogenases (HSDHs) enzymes catalyse the stereospecific oxidation of bile acid hydroxyl to oxo groups. Epimerisation of hydroxyl groups requires both  $\alpha$ - and  $\beta$ -HSDHs and occurs via stereospecific oxidation followed by stereospecific reduction of the ensuing oxo group(18). These metabolic reactions by HSDHs may neutralize the activity of antimicrobial BAs and provide an energy source for the indigenous microbiota(486). HSDH enzymes are evident in several intestinal bacteria, including species of the genera *Ruminococcus* and *Bacteroides*, both of which were found to have a higher abundance in our IBS cohort, with the latter being abundant in both IBS and BAD cohorts(18). Therefore, given this overlap of microbial communities, it is difficult to ascertain their role in the dysbiosis observed within each disease.

Gram-negative bacteria are believed to inherently be more resistant to bile than Gram-positive bacteria, which our data partially suggests by the increased abundance of *Prevotella*, *Verrucomicrobia* and *Bacteroides* seen(17). However, based on studies of the effect of bile salts on a collection of 38 strains of *Lactobacillus*, where varying degrees of bile tolerability were found, it has also been suggested that bile tolerance is strain-specific and variability may occur in microbial members of the same species or genus(17). This would therefore explain why an abundance of

the gram-positive taxa, Bifidobacteria and Lachnospiraceae, was also detected. The biological significance of the complex interaction between the gut commensals, host and bile acid metabolism is presently speculative. As well as benefits to the host through direct 'detoxification' and facilitation of bile acid absorption, it is hypothesised that the gut microbiota also gain from this interaction through the harvesting of energy and nutrients, which confer a survival benefit over other microbes(338).

## **2.5 Conclusions**

An important limitation of this study is the significant heterogeneity within the patient cohorts, which is likely to account for the inconsistencies in the reported data and ours. This is secondary to high inter-subject variations (phenotypic heterogeneity), differences in molecular techniques employed in analysing the intestinal microbiota, potential confounding environmental factors (e.g. diet, medications) and time point of sampling during fluctuating symptoms (periods of remission, relapse and changes in bowel function) as studies suggest IBS gut microbiomes are less stable(292, 402, 403). Although the adult intestinal microbiome is considered to be stable, environmental factors including antibiotics, diet, alcohol and smoking status, may all contribute to modified bile acid metabolism(378). Nevertheless, this is the first study to report observations of the microbiome milieu in those with BAD compared with those with IBS in whom BAD has been excluded.

There is clearly paucity in knowledge, especially of the role of indigenous microbiome in both BAD and IBS. Further large-scale, prospective studies with matching analytic approaches and patient cohorts are required to determine if the global composition of the intestinal microbiome, rather than the presence of single microbes, are relevant in the pathogenesis of both conditions. The current diagnostic tool of choice for BAD, the tauroselcholic [75 selenium] acid (SeHCAT) scan, is limited by its cost and availability, as is the management of this disease with empirical use of bile acid sequestrants, which often results in low compliance due to unpalatability.



Therefore, there is significant clinical need to ascertain the role of the gut microbiome as a potential non-invasive diagnostic biomarker in BAD, which may open up new avenues in creating management strategies through manipulation of the gut flora.

# **Chapter 3:**

## **Measurement of**

### **SCFAs in BAD**

### **and IBS-D**

## 3.1 Introduction and Aims

### 3.1.1 Introduction

Short chain fatty acids (SCFAs) are produced by anaerobic bacterial fermentation of non-digestible carbohydrates and certain amino acids and are the end products of metabolism. The three primary SCFAs produced are acetate, propionate and butyrate (usually in a 3:1:1 ratio) with a total concentration of 50-150 mM in the colon(488). The composition of the microbiome and environmental conditions, including hydrogen partial pressure, available substrates and pH all influence the synthesis of the different fermentation products(488). Modifications in intestinal fermentation can result in physiological abnormalities such as altered motility and excessive intraluminal gas production in IBS(489). The role of SCFAs in other gastrointestinal conditions, including ulcerative colitis and diversion colitis, has already been implicated.

SCFAs are integral to host health and provide 5-10% of human basal energy requirements. They have many important functions and are essential to intestinal homeostasis through a range of mechanisms including exerting regulatory pro-absorptive/anti-secretory effects on colonic transepithelial ion transport, mediation of colonic tropism through stimulating cell proliferation in the crypts, increasing visceral blood flow to improve tissue oxygenation, bearing anti-carcinogenic properties such as inducing apoptosis, improving nutrient digestion in slowing the passage of food in the upper gastrointestinal tract and maintaining the intestinal wall defense barrier(241). The concentration of butyrate in the systemic circulation is low as it is preferentially used as an energy source by intestinal epithelial cells(490). Propionate is largely metabolised in the liver and only acetate attains relatively high serum concentrations (0.10-0.15 mM)(488).

There is evidence to support the aetiological role of the intestinal microbiota in IBS. In addition, it is vital that the function of the microbiota is also

considered, as this may be as important as the phylotype in the initiation of disease. Microbial metabolites, in the form of SCFAs may be used as broad markers of microbial functionality, mostly associated with diet originating metabolites. Given the limited use of the SeHCAT scan in clinical practice, there is a significant, unmet requirement for the development of a novel biomarker test to diagnose BAD with this void being potentially fulfilled by utilising SCFAs.

To date, there is minimal data with none at all that examines the metabolic activity in terms of SCFA production in IBS-D and BAD, respectively. It is conceivable that the synthesis of SCFAs may be different in these two conditions given that we would expect differences in the various aspects that are known to determine SCFA concentration in the lumen, including gastrointestinal transit time, motility and physiology, gut microbiota composition and the amount and type of fermentable substrate ingested (if patients have already modified their diet to help with symptoms). To our knowledge, this is the first study of its kind to examine the concentrations of SCFAs in BAD and compares them to patients with IBS-D.

### **3.1.2 Aims**

Expanding on our previous study of delineating the intestinal microbial composition in IBS-D and BAD patients, the primary aim of this study was to understand the functional role of the microbiota in the aetiology of IBS and BAD. This was undertaken by measuring major bacterial metabolites in the faecal samples of both cohorts of patients.

## **3.2 Materials and Methods**

### **3.2.1 Recruitment and Sample Collection**

#### ***Ethical Approval***

Patients were recruited as part of the FAMISHED (Food and Fermentation using Metagenomics in Health and Disease) study. Scientific and ethical approval was acquired from the local Research and Development Office as well as Warwickshire Ethical committee ref: 09/H1211/38. Written informed consent was obtained from all participants in the study.

#### ***Study Participants***

Patients from the Nuclear Medicine department at University Hospitals Coventry and Warwickshire (UHCW NHS Trust) were recruited from December 2010 to July 2016 after being referred for a SeHCAT scan via attending a gastroenterology clinic with chronic diarrhoea. 20 patients with Rome III criteria IBS-D and 20 patients with BAD participated in the study.

Demographic and clinical data including age, gender, ethnicity, BMI, C-reactive protein (CRP – marker of inflammation) and faecal calprotectin levels (FCP - a measurement of the protein calprotectin in the stool, which is elevated during intestinal inflammation therefore used to ensure patients with IBD are in remission) for patients with type 1 BAD and co-existing IBD, SeHCAT result, severity and type of BAD were collected.

#### ***Sample Collection***

Stool samples were collected in standard specimen collection bottles in the nuclear medicine department and stored at -80°C within two hours of collection. They were transferred to the University of Glasgow in dry ice (-78°C) within a polybox and then stored at -70°C.

### ***Inclusion and Exclusion Criteria***

To be included in this cross-sectional study, both male and female participants were required to meet the following criteria: (1) have chronic diarrhoea, (2) diagnosis of BAD based on a SeHCAT retention value of  $\leq 15\%$ , (3) patients with IBD (inflammatory bowel disease) may only be included if type 1 BAD is present, (4) patients who have had a previous cholecystectomy may only be included if type 3 BAD is present.

Participants were excluded from the study if they suffered from coeliac disease, IBD (if type 1 BAD is not present), active IBD (defined as FCP  $> 50$  mg/kg stool or CRP  $> 11$  mg/L at the time of the SeHCAT scan), colorectal cancer or had been on antibiotics/probiotics in the last three months.

### **3.2.2 Power Calculation**

This was an exploratory pilot study to investigate SCFA concentrations in BAD and IBS-D. Assuming the standardized difference is 0.5, which corresponds to a moderate difference, a power of 31% (based on sample size) for a 2-sided test at 20% significance was deemed reasonable.

### **3.2.3 Molecular Data Collection**

Analysis was undertaken at the University of Glasgow, School of Medicine, by Dr Konstantinos Gerasimidis, Margarita Kokkorou and Vaios Svolos. This required special expertise available in very few centres, hence why this work was unable to be conducted locally. Short chain fatty acids (C2-C8) and branched chain fatty acids (isobutyrate, isocaproic and isovaleric) were measured utilising a method outlined by Laurentin and Edwards (2004) as well as the use of gas chromatography in ether extracts.

### **3.2.4 Preparation of Faecal Samples Prior to SCFA Analysis**

0.8 – 1.5g of faecal specimens were placed into a 7ml bijoux tube with an equal volume of 1M NaOH. SCFAs are susceptible to oxidation therefore conversion to their salt form by substitution of their free carboxylic hydroxyl group by divalent bonds is required to stabilize the samples. This is conducted in strong alkaline solution to reduce their volatility, preserve from oxidation and prevent continuing bacterial metabolic activity.

Pre-weighed small beads or magnetic stirrer were used in specimens of firmer consistency to optimize homogenization. Each tube was mixed by vortexing for one minute. This procedure was repeated a further two times and the samples were then stored at -20°C until required.

Moisture content was measured the day prior to SCFA analysis with the samples being freeze dried for 24 hours using Edwards apparatus (Freezer Dryer Micro Modulyo). The sample dry weight was calculated and the moisture content was expressed as a percentage of water per mass of stool specimen.

### **3.2.5 Identification of SCFAs by Gas Chromatography**

An Agilent 7890 gas chromatograph (ThermoQuest Ltd, Manchester, UK), which included a flame ionization detector (250°C) and a Zebron ZB-Wax capillary column (15m x 0.53mm x 1 µm film thickness) made of polyethylene glycol (catalogue No. 7EK-G007, Phenomenex, Cheshire, UK) was used to calculate the SCFAs present in the specimens. Nitrogen (30ml/min) was used as the carrier gas.

100µl of concentrated orthophosphoric acid and 100µl of internal standard solution (86.1 mmol/l 2-Ethylbutyric acid) were added to 300µl of distilled water, which contained 50µg freeze dried faeces. The mixture was vortexed for 15 seconds and 1.5ml diethyl ether was then added to each tube. The sample was vortexed for 1 minute with the ether phase supernatant being

recovered and pooled into a clean tube. This process was repeated a further two times. The pooled extract (1ul) was injected (injector temp 230°C, splitless) onto the column. The temperature of the column was initiated at 80°C for 1 minute, increasing by 15°C per minute to a final temperature of 210°C.

For calibration, an external standard (pH 8) consisting of 166.5 µmol/l acetic, 135 µmol/l propionic, 113.5 µmol/l isobutyric, 113 µmol/l butyric, 97.9 µmol/l isovaleric, 97.9 µmol/l valeric, 86.1 µmol/l hexanoic, 76.8 µmol/l heptanoic and 69.3 µmol/l octanoic was used. The external standard was run once more and after every 12 samples, the coefficient of covariance was calculated. The reagents used were Analytical Reagent Grade and were supplied by Sigma-Aldrich Company Ltd (Dorset, UK), except for acetic acid (glacial), which was supplied by Fisher Scientific (Loughborough, UK). All reagents were stored in universal tubes wrapped in aluminium foil or in dark bottles and left in room temperature before use.

To minimize inter-assay error, all serial samples from each patient were analysed in the same run. Due to evaporation of the very volatile SCFAs, time effect was accounted for by extracting and analysing each sample in duplicate (two different extractions) and in reverse order, to improve accuracy of results. Unless there was a widened variance, the results from the two extracts were averaged. To ensure repeatability of the assay and intra-assay comparison of the results between different runs, a quality control sample of thoroughly homogenized freeze-dried stock faecal material was included at the initiation and termination of each run.

In collaboration with Dr Konstantinos Gerasimidis's research group at the School of Medicine, University of Glasgow, healthy controls (HCs) were also recruited for this study. In total, 26 patients participated and none of them had any evidence of chronic disease, were on regular medications (including probiotics and prebiotics), pregnant or taken antibiotics in at least 3 months. Their stool samples were analysed within 2 days of the samples from the patients with BAD and IBS-D.



### **3.2.6 Statistical Analysis**

Statistical analyses were performed using SPSS Statistics version 24. Normality of the distribution of the data was assessed by using the Shapiro-Wilk test. For normally distributed data, comparisons of the means were assessed using the Student's *t* test. The Mann-Whitney U test was used for data, which was not normally distributed. Given this is a pilot study, *p* values of less than or equal to 20% were considered as statistically significant ( $p \leq 0.20$ ). Reported *p* values and 95% CI are based on two-sided tests.

When data was normally distributed in all 3 patient cohorts, comparison of means was undertaken by analysis of variance (ANOVA). Kruskal-Wallis analysis was used to determine comparison of the means in the 3 groups where data was not normally distributed.

The data are presented with means, standard deviations (SD), mean differences, 95% confidence intervals (CI) and *p*-values.

## **3.3 Results**

### **Demographic Data**

Demographics of the 66 participants (20 BAD: 20 IBS-D: 26 HCs) are seen in the table 14. The 40 IBS-D and BAD patients were matched for age (up to 5 years) and sex.

**Table 14: Demographic data of the study participants**

	<b>BAD (n=20)</b>	<b>IBS-D (n=20)</b>	<b>HCs (n=26)</b>
<b>Age (years)</b>	54.4 ± 12.3	55.4 ± 13.1	24.3 ± 3.0
<b>Gender</b>	17/20 (85%) F 3/20 (15%) M	17/20 (85%) F 3/20 (15%) M	15/26 (57.7%) F 11/26 (42.3%) M
<b>Ethnicity</b>	18/20 (90%) Caucasian 2/20 (10%) Indian	19/20 (95%) Caucasian 1/20 (5%) Indian	25/26 (96.2%) Caucasian 1/26 (3.8%) African
<b>BMI</b>	28.5 ± 8.1	27.3 ± 5.1	22.4 ± 2.6
<b>SeHCAT value (%)</b>	7 ± 4	39 ± 0.1	N/A
<b>Severity of BAD</b>	4/20 (20%) Mild 6/20 (30%) Moderate 10/20 (50%) Severe	N/A	N/A
<b>Type of BAD</b>	2/20 (10%) Type 1 14/20 (70%) Type 2 4/20 (20%) Type 3	N/A	N/A

- 35 of the 40 patients (17 with BAD and 18 with IBS-D) had a body mass index (BMI) recorded.
- Results expressed as means ± SD.

The BAD cohort of patients (17 female and 3 male) aged 35 to 79 years had a mean BMI of 28.5, defining them as being overweight. The mean SeHCAT value was 7%, which is consistent with moderate BAD. Of the 20 patients, 4 (20%) had mild, 6 (30%) had moderate and 10 (50%) had severe severity of disease respectively. Most (14 – 70%) patients had type 2 BAD, 2 (10%) patients had type 1 and 4 (20%) had type 3 BAD.

The IBS-D cohort of patients (17 female and 3 male) aged 32 to 77 years had a mean BMI of 27.3, defining them as being overweight. The mean SeHCAT value was 39%, which excluded them from having BAD.

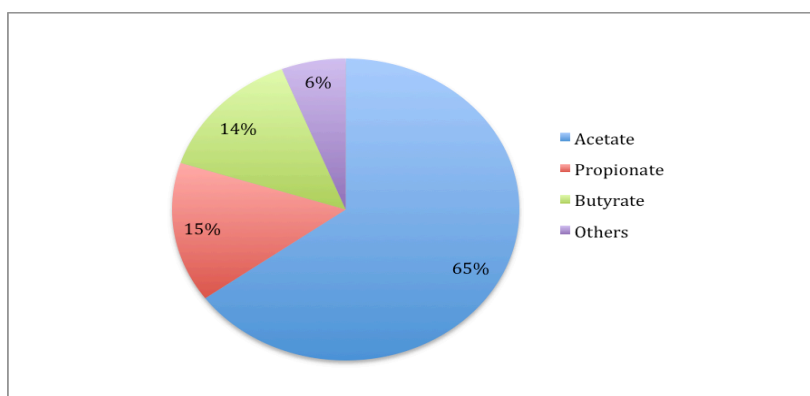
The HCs (15 female and 11 male) aged 21 to 35 years had a mean BMI of 22.4, defining their weight as normal.

### SCFAs in each group

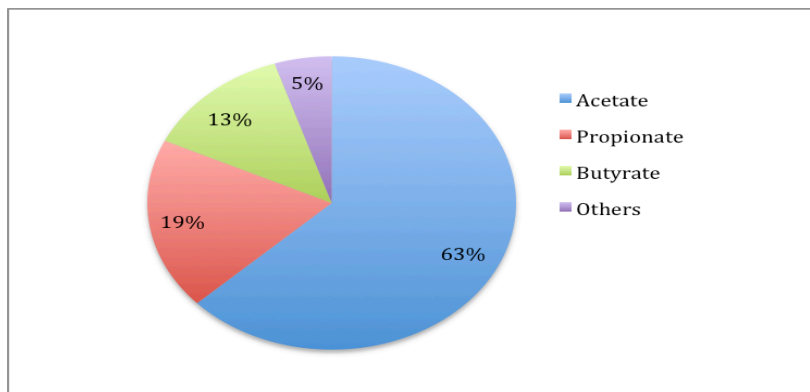
Below are three pie charts demonstrating the SCFA profile (the three main SCFAs of acetate, propionate and butyrate with the remaining SCFAs classified as 'others') of the HCs (figure 8) and each disease group (Figures 9 and 10 – BAD and IBS-D, respectively).

As expected with each group, acetate is responsible for the majority of SCFAs, however the ratio of acetate to propionate and butyrate is 4:1:1, a slight increase from the ratio quoted in the literature, as discussed earlier (22-29). The proportion of propionate (19%) in BAD exceeds that of the proportions found in HCs (15%) and IBS-D (14%).

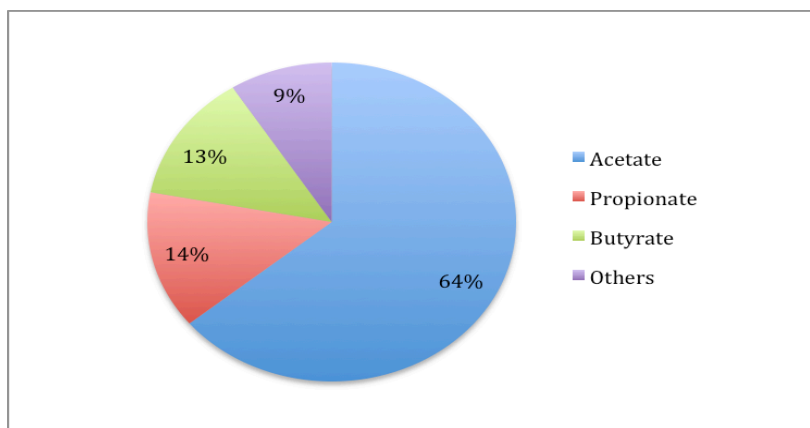
**Figure 8: Faecal SCFA profile in HCs**



**Figure 9: Faecal SCFA profile in BAD**



**Figure 10: Faecal SCFA profile in IBS-D**



### **Comparisons of SCFAs between the 3 groups**

Below are three tables demonstrating the water content and proportional ratios (%) and concentrations of the major bacterial metabolites in the faecal samples of patients with BAD/IBS-D and HCs (Table 15), BAD and IBS-D (Table 16), BAD and HCs (Table 17) and IBS-D and HCs (Table 17).

**Table 15: Comparison of the mean concentrations and proportional ratios (%) of the major bacterial metabolites in the faecal samples of all 3 cohorts of patients: BAD, IBS-D and HCs.**

SCFA	Mean (BAD)	Mean (IBS-D)	Mean (HC)	p-value
Faecal water content (%)	75.4%	74.1%	68.1%	<b>0.05</b>
Acetic acid (C2), $\mu\text{mol/g}$	464.2	381.9	343.8	0.29
% Acetic acid (C2)	63.1%	64.4%	64.7%	0.87
Propionic acid (C3), $\mu\text{mol/g}$	156.6	95.8	81.6	<b>0.11</b>
% Propionic acid (C3)	19.0%	14.4%	15.0 %	<b>0.20</b>
Butyric acid (C4), $\mu\text{mol/g}$	116.9	87.5	75.0	0.98
% Butyric acid (C4)	12.9%	13.0%	13.5 %	0.48
Valeric acid (C5), $\mu\text{mol/g}$	10.9	11.6	12.9	0.44
% Valeric acid (C5)	1.5%	2.3%	2.3%	<b>0.04</b>
Caproic acid (C6), $\mu\text{mol/g}$	1.7	5.5	5.4	<b>0.14</b>
% Caproic acid (C6)	0.1%	1.1%	0.94 %	<b>0.01</b>
Heptanoic acid (C7), $\mu\text{mol/g}$	0.2	0.7	0.73	<b>0.17</b>
% Heptanoic acid (C7)	0.03%	0.3%	0.13 %	<b>0.11</b>
Octanoic acid (C8), $\mu\text{mol/g}$	0.5	0.4	0.32	0.91
% Octanoic acid (C8)	0.2%	0.1%	0.06 %	0.85
Iso-butyric acid (iC4), $\mu\text{mol/g}$	7.9	9.3	8.3	0.57
% Iso-butyric acid (iC4)	1.2%	2.2%	1.7%	<b>0.07</b>
Iso-valeric acid (iC5), $\mu\text{mol/g}$	10.0	9.1	8.25	0.68
% Iso-valeric acid (iC5)	1.6%	2.3%	1.7%	0.70
Iso-caproic acid (iC6), $\mu\text{mol/g}$	0.7	0.3	0.4	<b>0.01</b>
% Iso-caproic acid (iC6)	0.1%	0.1%	0.07 %	<b>0.11</b>
Total SCFA, $\mu\text{mol/g}$	769.6	602.2	536.6	0.30

Values highlighted in bold signify statistically significant results ( $p \leq 0.20$ ).

**Table 16: Comparison of the mean concentrations and proportional ratios (%) of the major bacterial metabolites in the faecal samples of patients with BAD and IBS-D**

SCFA	Mean (BAD)	Mean (IBS-D)	Mean difference (95% CI)	p-value
Faecal water content (%)	75.4%	74.1%	1.3 (-4.8 to 7.4)	0.67
Acetic acid (C2), $\mu\text{mol/g}$	464.2	381.9	82.3 *	<b>0.14</b>
% Acetic acid (C2)	63.1%	64.4%	1.3 (-8.9 to 6.4)	0.75
Propionic acid (C3), $\mu\text{mol/g}$	156.6	95.8	60.8 *	<b>0.11</b>
% Propionic acid (C3)	19.0%	14.4%	4.6 (-0.6 to 9.8)	<b>0.08</b>
Butyric acid (C4), $\mu\text{mol/g}$	116.9	87.5	29.4 *	0.39
% Butyric acid (C4)	12.9%	13.0%	0.1 *	0.31
Valeric acid (C5), $\mu\text{mol/g}$	10.9	11.6	0.5 *	0.80
% Valeric acid (C5)	1.5%	2.3%	0.8 *	<b>0.04</b>
Caproic acid (C6), $\mu\text{mol/g}$	1.7	5.5	3.8 *	<b>0.14</b>
% Caproic acid (C6)	0.1%	1.1%	1.0 *	<b>0.00</b>
Heptanoic acid (C7), $\mu\text{mol/g}$	0.2	0.7	0.5 *	<b>0.16</b>
% Heptanoic acid (C7)	0.03%	0.3%	0.27 *	<b>0.10</b>
Octanoic acid (C8), $\mu\text{mol/g}$	0.5	0.4	0.1 *	0.66
% Octanoic acid (C8)	0.2%	0.1%	0.1 *	0.57
Iso-butyric acid (iC4), $\mu\text{mol/g}$	7.9	9.3	1.4 (-4.6 to 1.8)	0.38
% Iso-butyric acid (iC4)	1.2%	2.2%	1.0 *	<b>0.06</b>
Iso-valeric acid (iC5), $\mu\text{mol/g}$	10.0	9.1	0.9 *	0.99
% Iso-valeric acid (iC5)	1.6%	2.3%	0.7 *	0.43
Iso-caproic acid (iC6), $\mu\text{mol/g}$	0.7	0.3	0.4 *	<b>0.10</b>
% Iso-caproic acid (iC6)	0.1%	0.1%	0 *	0.58
Total SCFA, $\mu\text{mol/g}$	769.6	602.2	167.4 *	<b>0.17</b>

(\*Mann-Whitney U test used and hence no CI)

Values highlighted in bold signify statistically significant results ( $p \leq 0.20$ ).

**Table 17: Comparison of the mean concentrations and proportional ratios (%) of the major bacterial metabolites in the faecal samples of patients with BAD and HCs as well as in patients with IBS-D and HCs.**

SCFA	Mean (SD) BAD	Mean (SD) HC	Mean difference (95% CI)	p-value	Mean (SD) IBS-D	Mean (SD) HC	Mean difference (95% CI)	p-value
Faecal water content (%)	75.4% (10.1)	68.1% (8.9)	7.3 (2.7 to 11.9)	<b>0.00</b>	74.1% (5.0)	68.1%	6.0 (1.8 to 10.2)	<b>0.00</b>
Acetic acid (C2), $\mu\text{mol/g}$	464.2 (250.8)	343.8 (109.8)	120.4 *	0.24	381.9 (255.1)	343.8	38.1 *	0.81
% Acetic acid (C2)	63.1% (13.1)	64.7% (4.8)	-1.5 (-7.1 to 4.1)	0.59	64.4% (10.7)	64.7%	-0.3 (-5.0 to 4.5)	0.91
Propionic acid (C3), $\mu\text{mol/g}$	156.6 (135.9)	81.6 (37.8)	75.0 *	<b>0.04</b>	95.8 (76.2)	81.6	14.2 *	0.88
% Propionic acid (C3)	19.0% (9.5)	15.0% (3.4)	4.0 *	0.12	14.4% (6.4)	15.0%	0.6 *	0.52
Butyric acid (C4), $\mu\text{mol/g}$	116.9 (135.5)	75.0 (35.5)	41.9 *	0.98	87.5 (66.0)	75.0	12.5 (-17.9 to 43.0)	0.41
% Butyric acid (C4)	12.9% (8.1)	13.5% (3.8)	-0.57 (-4.2 to 3.0)	0.75	13.0% (4.6)	13.5%	0.5 *	0.91
Valeric acid (C5), $\mu\text{mol/g}$	10.9 (11.2)	12.9 (9.0)	2.0 *	0.24	11.6 (6.0)	12.9	1.3 *	0.84
% Valeric acid (C5)	1.5% (1.3)	2.3% (1.1)	0.8 *	<b>0.02</b>	2.3% (1.4)	2.3%	0 *	0.91
Caproic acid (C6), $\mu\text{mol/g}$	1.7 (0.9)	5.4 (5.3)	3.7 *	<b>0.04</b>	5.5 (5.4)	5.4	0.1 *	1.0
% Caproic acid (C6)	0.1% (0.3)	0.94% (0.8)	0.84 *	<b>0.0</b>	1.1% (1.3)	0.94%	0.16 *	0.88
Heptanoic acid (C7), $\mu\text{mol/g}$	0.2 (0.2)	0.73 (1.0)	0.53 *	<b>0.05</b>	0.7 (1.3)	0.73	0.03 *	0.69
% Heptanoic acid (C7)	0.03% (0.05)	0.13% (0.15)	0.1 *	<b>0.03</b>	0.3% (0.4)	0.13%	0.17 *	0.81
Octanoic acid (C8), $\mu\text{mol/g}$	0.5 (1.7)	0.32 (0.72)	0.18 *	0.78	0.4 (0.9)	0.32	0.08 *	0.91
% Octanoic acid (C8)	0.2% (0.58)	0.06% (0.11)	0.14 *	0.69	0.1% (0.25)	0.06%	0.94 *	0.93
Iso-butyric acid (iC4), $\mu\text{mol/g}$	7.9 (5.4)	8.3 (3.1)	-0.36 (-2.9 to 2.2)	0.78	9.3 (4.5)	8.3	1.0 (-1.2 to 3.3)	0.35
% Iso-butyric acid (iC4)	1.2% (0.9)	1.7% (0.7)	-0.43 (-0.9 to 0.05)	0.08	2.2% (1.7)	1.7%	0.5 *	0.49
Iso-valeric acid (iC5), $\mu\text{mol/g}$	10.0 (5.9)	8.25 (3.7)	1.75 *	0.43	9.1 (4.9)	8.25	0.9 (-1.7 to 3.4)	0.48
% Iso-valeric acid (iC5)	1.6% (0.9)	1.7% (0.9)	0.1 *	0.79	2.3% (1.9)	1.7%	0.6 *	0.55
Iso-caproic acid (iC6), $\mu\text{mol/g}$	0.7 (1.1)	0.4 (0.6)	0.3 *	<b>0.00</b>	0.3 (0.2)	0.4	0.1 *	0.32
% Iso-	0.1%	0.07%	0.03	<b>0.05</b>	0.1%	0.07%	0.03	<b>0.13</b>

caproic acid (iC6)	(0.3)	(0.1)	*			(0.05)		*	
Total SCFA, $\mu\text{mol/g}$	769.6 (457.5)	536.6 (179.1)	232.9 (35.4 to 430.4)	<b>0.02</b>		602.2 (387.3)	536.6 (179.1)	65.6 (-107.0 to 238.2)	0.45

(\*Mann-Whitney U test used and hence no CI)

Values highlighted in bold signify statistically significant results ( $p \leq 0.20$ ).

Results stated as means and (SD).

## BAD versus IBS-D versus HCs

Between these three groups, there were statistical significant differences observed for the faecal water content (figure 11), concentrations of propionic, caproic, heptanoic and iso-caproic acids, as well as for the proportions of propionic, valeric, caproic, heptanoic, iso-butyric and iso-caproic acids.

## BAD versus IBS-D

A greater concentration of total SCFAs was observed in BAD compared with IBS-D (769.6  $\mu\text{mol/g}$  in BAD versus 602.2  $\mu\text{mol/g}$  in IBS,  $p = 0.17$ ), which reached statistical significance (figure 12). Of the SCFAs, acetic acid was the metabolite with the greatest concentration observed in both BAD and IBS-D (464.2  $\mu\text{mol/g}$  in BAD versus 381.9  $\mu\text{mol/g}$  in IBS-D), which accounted for 63-64% of the total composition of SCFAs (figure 13). In both BAD and IBS-D, propionic acid and butyric acid represented the second and third greatest proportion of SCFAs respectively, with propionic acid accounting for 14-19% and butyric acid for 13% of the total composition of SCFAs. Statistical significance was achieved for greater concentrations of acetic acid ( $p = 0.14$ ), concentrations ( $p = 0.11$ ) and proportions ( $p = 0.08$ ) of propionic acid in BAD patients compared to those in IBS-D.

Statistical significance for the difference in concentrations and proportions of SCFAs demonstrated in BAD and IBS-D was attained for % valeric acid, caproic acid, % caproic acid, heptanoic acid, % heptanoic acid, %isobutyric acid and iso-caproic acid. With the exception of the concentration of iso-caproic acid, these metabolites were observed to be present in greater



quantities/proportions in IBS-D patients compared to those with BAD. These metabolites represented minimal quantities (0.03 to 2.3%) of the total composition of SCFAs. Please see table 3 for full details.

### **BAD versus HCs**

A greater concentration of total SCFAs was observed in BAD compared with HCs (769.6 $\mu$ mol/g in BAD versus 536.6 $\mu$ mol/g in HCs), which reached statistical significance ( $p = 0.02$ ). The proportion of water in the faecal samples of those with BAD (75.4%) was statistically higher ( $p = 0.00$ ) compared to the faecal water proportion in patients with HCs (68.1%). Of the SCFAs, acetic acid was the metabolite with the greatest concentration observed in both BAD and HCs (464.2  $\mu$ mol/g in BAD versus 343.8  $\mu$ mol/g in HCs), which accounted for 63-65% of the total composition of SCFAs. In both BAD and HCs, propionic acid and butyric acid represented the second and third greatest proportion of SCFAs respectively, with propionic acid accounting for 15-19% and butyric acid for 13-14% of the total composition of SCFAs. Statistical significance was achieved for a greater concentration of propionic acid ( $p = 0.04$ ) in BAD (156.6 $\mu$ mol/g) compared to patients with HCs (81.6 $\mu$ mol/g) (figure 14).

Statistical significance for the difference in concentrations and proportions of SCFAs demonstrated in BAD and HCs was attained for % valeric acid ( $p = 0.02$ ), caproic acid ( $p = 0.04$ ), % caproic acid ( $p = 0.00$ ), heptanoic acid ( $p = 0.05$ ), % heptanoic acid ( $p = 0.03$ ), iso-caproic acid ( $p = 0.00$ ) and %iso-caproic acid ( $p = 0.05$ ). With the exception of the concentration and proportion of iso-caproic acid, these metabolites were observed to be present in greater quantities/proportions in HCs patients compared to those with BAD. These metabolites represented minimal quantities (0.07 to 2.3%) of the total composition of SCFAs. Please see table 4 for full details.

## IBS-D versus HCs

The proportion of water in the faecal samples of those with IBS-D (74.1%) was statistically higher compared to the faecal water proportion in patients with HCs (68.1%). Of the SCFAs, acetic acid was the metabolite with the greatest concentration observed in both IBS-D and HCs (381.9  $\mu\text{mol/g}$  in IBS-D versus 343.8  $\mu\text{mol/g}$  in HCs), which accounted for 64-65% of the total composition of SCFAs. In both IBS-D and HCs, propionic acid and butyric acid represented the second and third greatest proportion of SCFAs respectively, with propionic acid accounting for 14-15% and butyric acid for 13-14% of the total composition of SCFAs. With the exception of the concentrations of propionic acid in patients with IBS-D and HCs, these three main metabolites were present in similar concentrations and proportions in both cohorts and did not reach statistical significance.

There was a greater concentration of total SCFAs in patients with IBS-D (602.2  $\mu\text{mol/g}$ ) compared to HCs (536.6  $\mu\text{mol/g}$ ) but this did not reach statistical significance ( $p = 0.45$ ).

Figures 11-14: Percentage of faecal water (11), concentration of total SCFAs (12), concentration of acetic acid (13) and concentration of propionic acid (14) in BAD, IBS-D and HCs. Error bars indicate SDs. Brackets denote statistically significant results with  $p$  values.

**Figure 11: Percentage of faecal water**

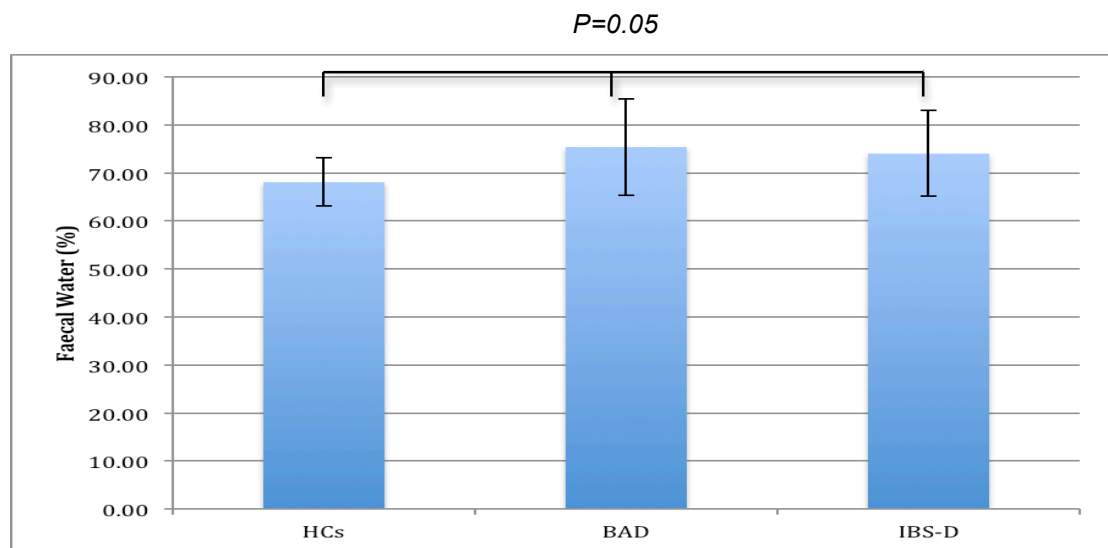


Figure 11 represents a statistical significance in the percentage of faecal water between the 3 groups.

**Figure 12: Concentration of total SCFAs**

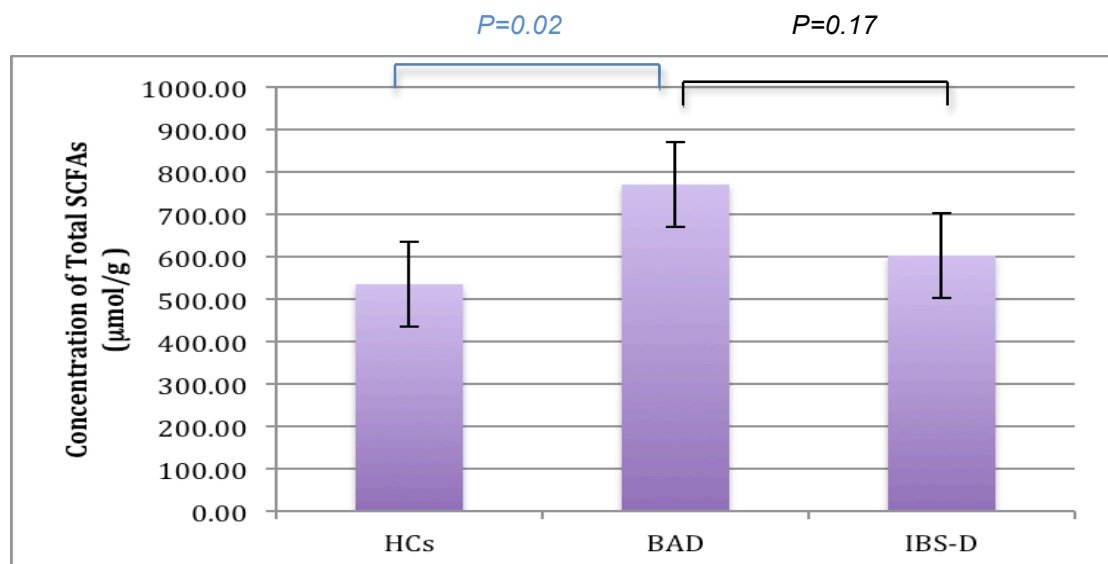


Figure 12 represents a statistical significance in the concentration of total SCFAs between the BAD and HCs groups as well as the BAD and IBS-D cohorts.

**Figure 13: Concentration of acetic acid**

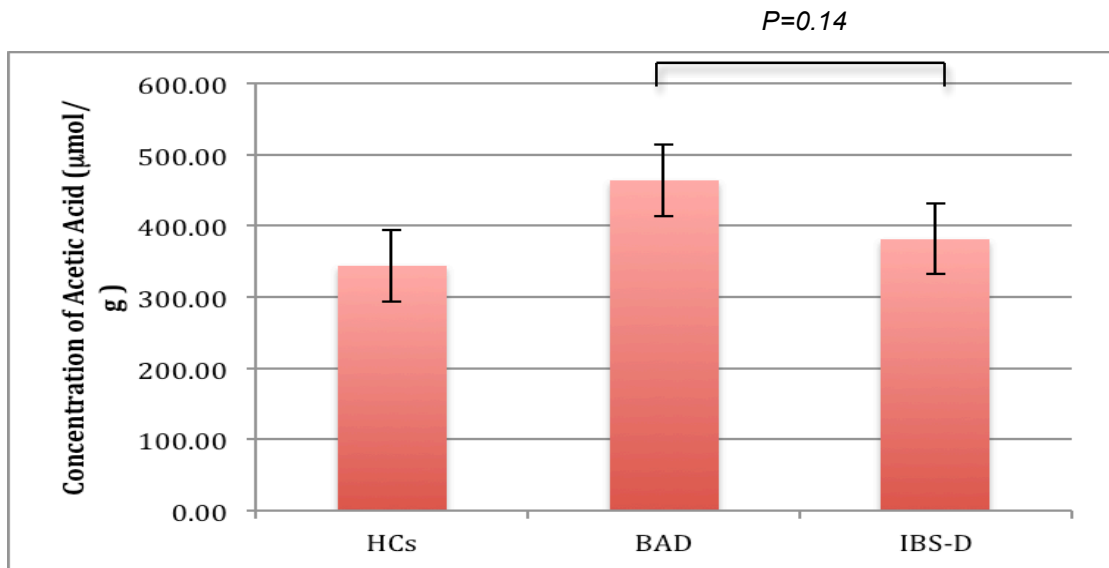


Figure 13 represents a statistical significance in the concentration of acetic acid between the BAD and IBS-D groups.

**Figure 14: Concentration of propionic acid**

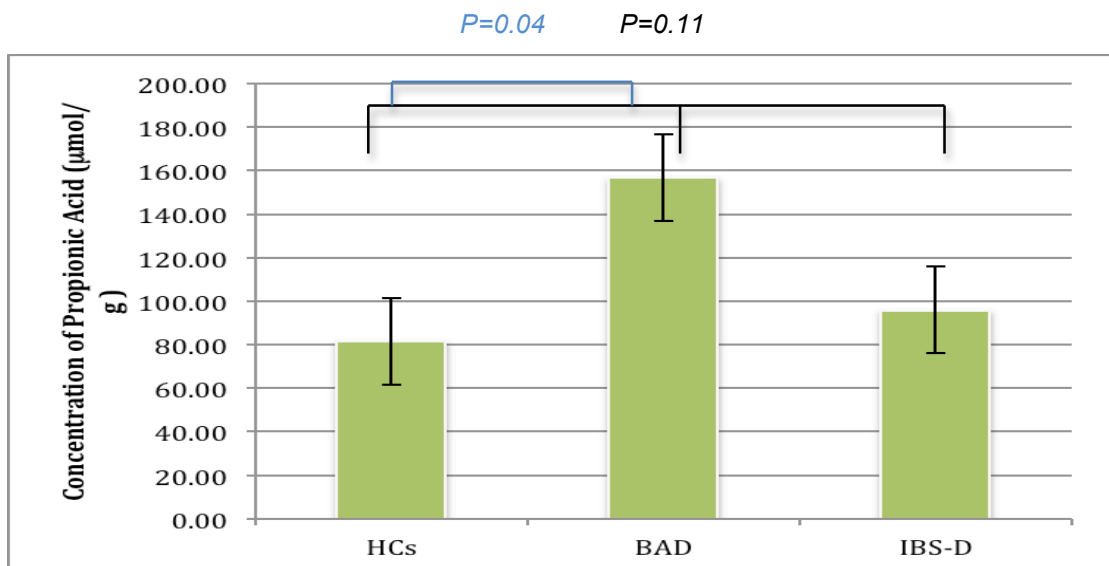


Figure 13 represents a statistical significance in the concentration of propionic acid between the BAD and HCs groups as well as between all 3 groups.

### 3.4 Discussion

Patients with IBS exhibit evidence of the condition being inflammatory in nature by demonstrating an imbalance of pro-inflammatory (TNF- $\alpha$ ) and anti-inflammatory (IL-10) cytokines and activation of the immune system(305, 491). SCFAs are recognised to possess anti-inflammatory effects as potential mediators involved in the effects of the intestinal microbiota on the intestinal immune function by acting on leucocytes and endothelial cells through two main mechanisms. These involve inhibition of histone deacetylase (HDAC) and activation of G-coupled protein receptors - GPCRs (GPR41 and GPR43). Murine studies have implicated GPR41 and GPR43 in chronic inflammatory disorders such as colitis, obesity, asthma and arthritis although studies remain contradictory(492). These receptors are located at multiple sites, including gut endocrine cells, adipose tissue, immune cells and pancreatic islets(242). *In vitro*, activation of these receptors by SCFAs induces neutrophil chemotaxis (SCFA-16). The affinity of SCFAs for these receptors differs in that GPR43 preferentially binds propionate and acetate, whereas GPR41 favorably binds propionate and butyrate with acetate being significantly less potent(239). Inhibition of HDAC activity allows for SCFAs to increase the acetylation of histone and non-histone proteins, which modulates gene expression to suppress the production of pro-inflammatory cytokines, including nitric oxide (NO), tumour necrosis factor (TNF- $\alpha$ ) and interleukin-6 (IL-6). Butyrate is the most potent inhibitor of HDAC activity with acetate being the least potent(239). In addition to this, propionate and butyrate induce the differentiation of regulatory T cells, which express FOXP3, a transcription factor and this plays a vital role in controlling intestinal inflammation(488). All these signaling pathways mediated by SCFAs contribute to control of active inflammation.

Given the anti-inflammatory role of SCFAs, as well as its production in concomitantly decreasing colonic pH, inhibiting pathogenic microorganisms

and increasing the absorption of nutrients, one would expect a more 'healthy' intestine with greater concentrations of SCFAs present(493). This was not demonstrated in our study and a statistically significant difference in the total number of SCFAs was observed with BAD patients having a greater concentration of SCFAs than patients with IBS-D and HCs. Patients with IBS-D were also observed to have a greater concentration of SCFAs than that in HCs. This may represent an upregulatory response of SCFAs to an inflammatory insult to the colon in disease. Another possibility is that the relative amount of individual SCFAs may be more important in the pathophysiology of BAD and IBS-D than the total amount of SCFAs. Studies in this area have generated conflicting results. For example, higher levels of acetic and propionic acid have been observed in IBS patients compared to HCs with raised levels correlating with significantly worse gastrointestinal symptoms, negative emotions and quality of life(296). Another study in patients with IBS-D observed decreased faecal SCFAs compared with HCs(489).

Interestingly, patients with constipation-predominant IBS (IBS-C) have been found to have significantly lower faecal SCFA levels compared to those with IBS-D, which suggests the possibility of an association between levels of SCFAs and colonic motility(489). The bowel transit rate may be a determinant of faecal SCFA concentration with one study demonstrating accelerated transit with the aid of senna resulting in an increase in SCFA concentrations whilst loperamide (slowing bowel transit) caused the inverse effect(494). This may help to explain the raised levels of SCFAs seen in both our disease cohorts (compared to HCs), both conditions resulting in increased bowel transit.

It is assumed that the overall rate of fermentation and therefore production of SCFAs is influenced by changes in diet with resistant starches being substrates for the intestinal microbiota and contribute the most to the production of SCFAs(240). However, it has been demonstrated that a diet increased in residual starch intake results in greater fermentation with increased breath H<sub>2</sub> production but surprisingly, the faecal concentration of

SCFAs remains unchanged, suggesting that any change is localized within the intestine and this may be secondary to changes in the composition of the gut microbiota(240). A high abundance of butyrate-producing Clostridial cluster IV bacteria is associated with diets enriched in resistant starches and is thought to contribute to increased SCFA production(495). Another important point to consider when measuring the production of SCFAs is the fact their concentrations are expressed per gram of the faecal sample with the total volume (mass) of faeces varying between and within the same participants.

The statistically significant increase in total SCFA, acetic acid and propionic acid concentrations observed in patients with BAD compared to those with IBS-D and HCs may also reflect altered colonic fermentation by the intestinal microbiota. IBS patients have demonstrated significantly higher counts of *Veillonella* and *Lactobacillus* with expression of significantly higher levels of SCFAs, acetic and propionic acid. Interestingly, high acetic acid levels were associated with increased scores of abdominal pain and bloating, with these symptoms being common in both BAD and IBS-D. These patients were diagnosed with IBS based on Rome criteria alone(296). BAD presents similarly to IBS-D, with 28% of patients misdiagnosed with IBS-D for BAD therefore it is conceivable that a significant proportion of these patients may have had their diagnosis revised after a SeHCAT scan(39). Further evidence of dysbiosis associated with altered SCFA levels has been observed in patients with small bowel bacterial overgrowth and colon-like microbiota having total SCFA concentrations that were four times greater than in healthy subjects(300). It is assumed therefore, that varying concentrations of individual SCFAs rather than an overall concentration of total SCFAs contributes to the genesis of disease.

Butyrate in particular, plays an important role in maintaining the colonic epithelium and is the preferred fuel used by colonocytes(496). Therefore, there should be an expected reduction in the level of butyrate in our disease cohorts compared to HCs but surprisingly, similar proportions were found

between the three patient groups. A possible explanation for this is the small sample size as butyrate-producing micro-organisms such as Ruminococcaceae, Clostridiales and Erysipelotrichaceae, have been found to be less abundant in patients with IBS-D(497). Butyrate appears to exert potent anti-inflammatory effects both *in vivo* and *in vitro* and blocks the development of DSS-induced experimental colitis via reducing concentrations of pro-inflammatory cytokines, including IL-6 and TNF- $\alpha$ (498). Therefore, lower levels of butyrate would help account for the possibly inflammatory component (which has been suggested in the literature) of IBS-D.

An 'intolerance' to complex carbohydrates in the diet may also account for the statistically significant greater faecal water content being observed in patients with both BAD and IBS-D compared to HCs. Osmotically active FODMAPs are poorly absorbed by the small bowel and result in net secretion of fluid with increased water delivery to the colon(220). Specifically in the IBS-D cohort, gluten has demonstrated increased small bowel permeability, which may result in increased fluid flux towards the lumen(153). This is further supported by evidence of an impaired epithelial barrier with disruption of the apical junctional complex integrity in the jejunal mucosa of patients with IBS-D(146). Therefore this suggests that this cohort of patients may be more vulnerable to the effects of carbohydrates in the diet although the underlying causative mechanisms have not been fully elucidated. The increased faecal water content in our disease cohorts is also a reflection of the reduced reabsorption of water in the colon secondary to increased transit time(252).

The literature states that proportions of SCFA in the lumen are approximately 60% acetate, 25% propionate and 10% butyrate and this ratio is mostly reflected in our data for both IBS and BAD patients(499). The proportion of propionic acid (14.4%) observed in the IBS-D cohort was almost half of the expected value seen in the HCs and significantly reduced compared to the proportion observed in patients with BAD. Interestingly, animal data has exhibited varying effects of differing SCFAs on colonic



motility. Butyrate demonstrated an increase in colonic propagation; propionate and to a lesser degree, acetate caused a decrease in propagation(500). Therefore, it can be extrapolated from this data that the role of propionate may be to reduce movement of bowel contents. This would fit with our data, where a reduction in levels of propionate observed could imply a tendency to the enhancement of propagation of colonic contents. This would clinically manifest as increased bowel frequency, a symptom hallmarking both IBS-D and BAD.

The difference between subjects with and without IBS has been observed to be highly statistically significant for the propionic acid/butyric acid ratio as well as subtracting the concentration of butyric acid from propionic acid (Prop-But). A value greater than 0.015 mmol/l was deemed as a suitable cut-off for a positive test for IBS, with a value less than -0.13 mmol/l excluding the diagnosis and reaching a sensitivity of 100%(305). This is not verified by our data as with the significantly reduced concentration of propionate observed in our IBS-D cohort compared to patients with BAD, the Prop-But value would be lower in our IBS-D patients thereby dismissing this as a potential diagnostic property of SCFAs in IBS-D.

The anti-inflammatory effects of specific SCFAs include acetate, propionate and butyrate decreasing the production of TNF- $\alpha$  by LPS-stimulated human neutrophils, as well as propionate and butyrate inhibiting the expression of pro-inflammatory mediators through diminution of NF- $\kappa$ B in rat neutrophils(239). Therefore, a reduction in the expected proportion of propionate observed in our IBS-D cohort compared to the general population as well as being statistically significantly lower than in patients with BAD may further implicate an inflammatory aetiological factor in this disease.

Significantly reduced concentrations and proportions of the minor and branched SCFAs (valeric acid, caproic acid, heptanoic acid and iso-butyric acid) in patients with BAD compared to those with IBS-D and HCs. These

acids are found in the faeces in small amounts, which our data reflects and likely arise from bacterial degradation of proteins to amino acids. Branched SCFAs are recognised in modulating glucose and lipid metabolism in primary adipocytes but their clinical significance, particularly in relation to colonic disease, remains unknown(501). As well as these amino acids serving as precursors for the synthesis of SCFAs, they have been found to contribute to metabolic disease, such as insulin resistance and obesity(502). The FXR has a recognised role in triglyceride metabolism through modulating free fatty acids oxidation and triglyceride clearance as well as hepatic de novo lipogenesis(503). A study has demonstrated a third of patients with primary BAD having hypertriglyceridaemia (this was also associated with increasing BMI) with hypertriglyceridaemia being linked to increased BA synthesis and production. These patients were also observed to have high FGF19 concentrations(504). Therefore, reduced concentrations of branched SCFAs in patients with BAD may be a reflection this disease being metabolic in aetiology with disturbed amino acid homeostasis accounting for the metabolic effects of FXR-induced triglyceride dysmetabolism as well as impaired FXR-mediated BA signalling.

In the general population, metagenomic analysis has demonstrated a bimodal distribution in the diversity of faecal microbiota with individuals either having a low gene count (LGC) or high gene count (HGC) as indicators of bacterial richness. The *Bacteroides* species dominate the LGC community and there is a reduction in the butyrate-producing Firmicutes(488). Therefore, the limitation of this study is that the underlying genetic make-up for the diversity in intestinal bacteria in our patient cohorts is unknown, which could be a contributory factor towards the SCFA profiles identified.

Increasing evidence, including our own, supports an association between diet, the microbiota and SCFA production with the presence of dysbiosis in these patients (this is further discussed in chapter 6). With this in mind, therapeutic modification of the intestinal microbiome in IBS via probiotics, non-absorbable antibiotics (Rifaximin) and faecal microbiota transplant is

gaining rapid interest. Current management strategies in BAD are unsatisfactory with 40-70% of bile acid sequestrant treatment being discontinued mainly due to poor palatability. Therefore, refining our understanding of the microbiota and its functionality in these conditions is vital in facilitating mechanistic insight into the composition of the microbiome as well as developing targeted, effective and individualized treatment for these patients. Patients with IBS-D are often managed with a low FODMAPs diet to reduce gastrointestinal symptoms such as pain, bloating and diarrhoea. FODMAPs are poorly absorbed carbohydrates, which are rapidly fermented by the gut microbiota therefore reduced concentrations of SCFAs would be expected from this diet(220).

Given that we assume the altered proportions of differing SCFAs in IBS-D and BAD compared to HCs is more likely to influence the development of disease rather than changes in the overall concentration of SCFAs, it is feasible that dietary interventions which increase/reduce particular SCFAs may be of benefit to these patients.

### **3.5 Conclusions**

In conclusion, we have demonstrated a difference in the total and relative amounts of SCFAs in IBS-D and BAD. To our knowledge, there has been no previous study to evaluate bacterial fermentation in BAD, as well as its comparison to IBS-D and HCs. The exact underlying aetiology in IBS-D and BAD remains unclear but it is plausible that SCFAs help shape the immune system and impact on intestinal homeostasis via inflammatory pathways, as well as affecting bowel transit and intestinal epithelial permeability. Greater research that focuses on the role of branched chain SCFAs is vital given the significant difference in their concentrations in the two disease cohorts. Further prospective studies are required to validate the use of SCFAs as a potential biomarker candidate and to further improve knowledge of their metabolic function in the pathophysiology of BAD.

# **Chapter 4:**

## **Measurement of**

### **Bile Acids in**

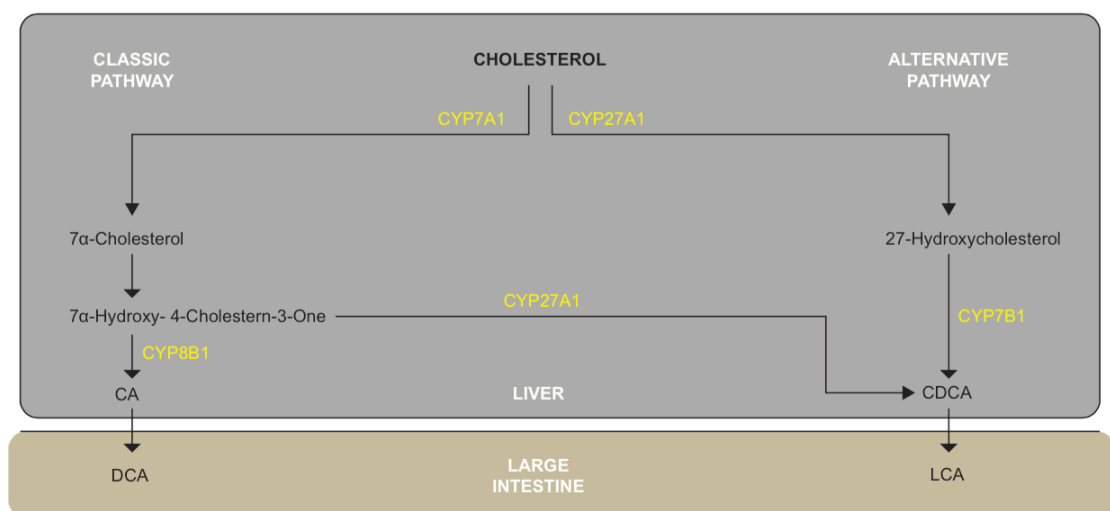
#### **BAD and IBS-D**

## 4.1 Introduction and Aims

### 4.1.1 Introduction

The enterohepatic cycle determines the composition of the bile acid (BA) pool with the BA pool size being defined as the total amount of BAs circulating in this cycle. The total BA pool of primary and secondary BAs is 2 to 5 grams and circuits the enterohepatic circulation 6 to 10 times daily, or 2 to 3 times per meal(505). The BA pool comprises of cholic acid (CA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) in an approximately 40:40:20 ratio(506). Within hepatocytes, the production of equal quantities of primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are derived mainly through the classical pathway from cholesterol and conjugated with glycine or taurine before being secreted into the bile and small intestine(506, 507). The cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) enzyme catalyses the first and rate-limiting step in this pathway and therefore influences the overall rate of BA production. The microsomal sterol 12 $\alpha$ -hydroxylase (CYP8B1) enzyme is responsible for 12 $\alpha$ -hydroxylation of the intermediate product of the CYP7A1 reaction, therefore this enzyme controls the CA:CDCA ratio in the BA pool (see figure 15)(506).

**Figure 15: Production of CA and CDCA through the classic and alternative pathways.**



These primary BAs undergo gut microbial modification through various enzymatic reactions: deconjugation, dehydrogenation, dehydroxylation and sulfation(506, 507). Once deconjugated, this results in the transformation of primary to secondary BAs: CA into DCA and CDCA into lithocholic acid (LCA)(370). Consequently, the proportions of the differing components in the BA pool vary in bile, serum and faeces with minor and larger quantities of primary and secondary BAs, respectively, evident in faeces(371). Serum levels of BAs reflect their uptake by the portal vein and re-conjugation during transit across the hepatocytes towards the bile(374). The specific transporter for the BAs, the apical sodium dependent bile acid transporter (ASBT), actively reabsorb conjugated BAs in the terminal ileum, while the secondary, unconjugated BAs are passively reabsorbed resulting in 5% of BAs being excreted in faeces(370). BAs leave the enterocyte via the OSTa/b transporter and sensing of the enterocyte BA pool by the farnesoid X receptor (FXR) is integral to BA homeostasis(19).

As a result of passive absorption through the colon as well as the incapability of the liver to 7 $\alpha$ -hydroxylate DCA and LCA back to their respective primary BAs, there is an accumulation of DCA (and LCA to a lesser extent) in the BA pool. Colonic transit time and pH also influence the quantity of DCA in the BA pool(371). At the 3-hydroxy position, LCA is sulfated and conjugated at C-24 by the liver before being excreted back into the bile. This resultant sulfated LCA is poorly reabsorbed from the intestine and does not accumulate in the enterohepatic circulation as it is lost in the faeces despite being deconjugated and desulfated to some extent by the intestinal microbiota(371).

In the colon, the secondary BAs, LCA and DCA, preferentially stimulate the G protein-coupled BA receptor (GPBAR1 or TGR5). Activation of TGR5 in the small intestine by DCA in mice inhibits gastric emptying and transit in both the small bowel and proximal colon, thereby promoting absorption of nutrients by acting as an 'ileal brake'(508). In a cohort of IBS-D patients, a TGR5 SNP demonstrated faster small bowel transit(364).

The control of BA synthesis and consequent motility effects of BAs in the colon is principally governed by the FXR, whose expression is highest in the liver and intestine (ileal enterocytes). FXR binds BAs and bears the strongest affinity for CDCA, with lower quantities of BAs such as LCA, displaying weaker affinity as FXR agonists. Activation of hepatic FXR results in expression of genes involved in BA metabolism, such as short heterodimer partner (SHP) transcription, which inhibits CYP7A1 and liver receptor homolog to inhibit BA synthesis. A second pathway, where ileal FXR activation causes upregulation of fibroblast growth factor 15 (FGF15) - the murine orthologue of human FGF19, and consequently fibroblast growth factor receptor 4 (FGFR4) in the hepatocyte also results in the inhibition of CYP7A1 and thus BA synthesis(509).

The relationship between bile acids and functional gastrointestinal diseases, such as IBS, has become more apparent in recent times(509). Given that the clinical phenotype of patients with bile acid diarrhoea (BAD) presenting with chronic diarrhoea is similar to those with IBS-D, it is not surprising that BAD is frequently misdiagnosed for IBS-D. An excess of 28% of patients meeting the Rome criteria for IBS-D actually demonstrate SeHCAT evidence of BAD(39). Interestingly, despite patients with primary BAD bearing an absence of intestinal disease, they demonstrate a similar increase in faecal BA loss as those with secondary, ileal BAD(59).

#### **4.1.2 Aims**

The aim of this study was to establish the composition of the BA pool in both BAD and IBS-D patient cohorts to determine if there are any differences in the serum and faecal bile acid profiles in both diseases.

## **4.2 Materials and Methods**

### **4.2.1 Recruitment and Sample Collection**

#### ***Ethical Approval***

Patients were recruited as part of the FAMISHED (Food and Fermentation using Metagenomics in Health and Disease) study. Scientific and ethical approval was acquired from the local Research and Development Office as well as Warwickshire Ethical committee ref: 09/H1211/38. Written informed consent was obtained from all participants in the study.

#### ***Study Participants***

Patients from the Nuclear Medicine department at University Hospitals Coventry and Warwickshire (UHCW NHS Trust) were recruited from January 2012 to August 2016 after being referred for a SeHCAT scan via attending a gastroenterology clinic with chronic diarrhoea. Serum and stool samples were retrieved from 15 patients with Rome III criteria IBS-D and 15 with BAD. Patients with IBS-D and BAD were sex and age-matched (up to 10 years). We endeavoured to yield both stool and serum samples from each patient but given that patients were matched for age and sex, this was only achievable in 5 patients with IBS-D and 5 with BAD.

Demographic and clinical data including age, gender, ethnicity, BMI, C-reactive protein (CRP – marker of inflammation) and faecal calprotectin levels (a measurement of the protein calprotectin in the stool, which is elevated during intestinal inflammation therefore used to ensure patients with IBD are in remission) for patients with type 1 BAD and co-existing IBD, SeHCAT result, severity and type of BAD were collected.

#### ***Sample Collection***

Stool and serum samples were all collected in the morning at 9am in standard specimen collection bottles in the nuclear medicine department and stored at -80°C within two hours of collection, after the serum was



homogenised. They were transferred to Paris, France on dry ice (-78°C) within a polybox and then stored at -80°C.

### ***Inclusion and Exclusion Criteria***

To be included in this cross-sectional study, both male and female participants were required to meet the following criteria: (1) have chronic diarrhoea, (2) diagnosis of BAD based on a SeHCAT retention value of  $\leq 15\%$  (3) patients with IBD (inflammatory bowel disease) may only be included if type 1 BAD is present, (4) patients who have had a previous cholecystectomy may only be included if type 3 BAD is present.

Participants were excluded from the study if they suffered from coeliac disease, IBD (if type 1 BAD is not present), active IBD (defined as FCP >50 mg/kg stool or CRP >11 mg/L at the time of the SeHCAT scan), colorectal cancer or had been on antibiotics/probiotics in the last three months.

## **4.2.2 Measurement of Faecal and Serum Bile Acids**

### ***Chemicals and Reagents***

Bile acid standards, CA, DCA, CDCA, LCA, ursodeoxycholic acid (UDCA), hyocholic acid (HCA), hyodeoxycholic acid (HDCA) as well as the corresponding glycine and taurine derivatives were acquired from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Acetic acid, ammonium carbonate and ammonium acetate were also obtained from Sigma-Aldrich. The 3-sulphate derivatives were kindly donated by Dr J. Goto (Niigita University of Pharmacy and Applied Life Sciences, Niigata, Japan). The internal standard solution (23-nor-5 $\alpha$ -cholanoic acid-3 $\alpha$ ,12 $\beta$ -diol) was purchased from Steraloids Inc., (Newport, RI, USA).

### ***Standard Solutions***

Standard stock solutions of the BAs were prepared in methanol (1mg/ml) and stored at -20°C in a sealed container. The stock solutions were then pooled together and further diluted in methanol to attain mixed calibration BA solutions with concentrations from 31.3  $\mu\text{g mL}^{-1}$  to 31.3 ng mL $^{-1}$ .

### ***Sample Preparation***

Using a Thermo Savant Speedvac (Thermo Fisher Scientific, Saint Herblain, France) (SPD 111V) coupled to a cooled vapour trap (RTV400; Thermo Fisher Scientific), 2  $\mu\text{l}$  of the internal standard solution was added to 0.1g of lyophilized faecal samples. At a ratio of 4ml/1ml of sample, 0.4 mol L<sup>-1</sup> ammonium carbonate was added to release the BA from the binding protein. The samples were then incubated at 60°C for 30 minutes. 4mls of water was added to the faecal samples and then homogenized in an Ultra-Turrax disperser (IMLAB, Lille, France) on two 30-second runs. Centrifugation (at 20,000 g for 20 minutes) followed by solid-phase extraction using reversed-phase silica Chromabond C18 cartridges (100 mg; Macherey-Nagel, Duren, Germany) ensured pre-analysis clean up. Samples were then loaded on to the cartridge and a vacuum manifold was used to undertake subsequent elution steps. The cartridge was rinsed with 20mls of water to discard salts and hydrophilic metabolites, followed by 10ml hexane to discard neutral lipids and then 20ml of water again. The BAs were eluted with 5ml of methanol. The methanol was then evaporated off under a nitrogen stream at 50°C. The residue was dissolved in 150  $\mu\text{l}$  methanol, of which 5  $\mu\text{l}$  was injected into the high-performance liquid chromatography– tandem mass spectrometry (HPLC–MS/MS) system.

### ***HPLC–MS/MS Analysis***

An analytical column (Pinnacle II C18, Restek, Lisses, France; 250 x 3.2 mm) with a 5  $\mu\text{m}$  silica particle (Restek) fitted on an HPLC binary pump (Agilent 1100; Agilent Technologies, Massy, France) was used for the chromatographic separation of BAs. The column was thermostated at 35°C. The mobile phases consisted of buffer (A) (ammonium acetate 15mmol/L, pH 5.3) and solvent (B) (methanol) at 63:35 (v/v). By increasing B in A from 65 to 95 (v/v) for 30 minutes, the BAs were eluted. To achieve separation, a flow rate between 0.3 and 0.5ml/minute for 30 minutes was set. Mass spectra were obtained using an API<sup>®</sup> 2000 Q-Trap (AB-Sciex, Concord, Ontario, Canada) equipped with a turbo ion-spray (ESI) source. Electrospray ionization was executed in negative mode with nitrogen as the

nebulizer gas. The temperature of the evaporation gas was fixed at 400°C. The ion-spray, declustering and entrance potentials were set at -4500, -60 and -10 V, respectively. The MS/MS detection was operated with unit/unit resolution in the multiple reaction monitoring (MRM) modes. For each transition, the dwell time of the ion trap was set at 70 ms. Analyst<sup>®</sup> software (version 1.4.2, AB-Sciex) was used to acquire the data.

#### **4.2.3 Statistical Analysis**

Statistical analyses were performed using JMP statistical software version 6.0, SPSS version 14.0 and XLSTAT 2012 (Addinsoft®, New York city, USA). For normally distributed data, comparisons of the means were assessed using the Student's *t* test. The Mann-Whitney U test was used for data, which was not normally distributed. The total primary BAs is the sum of CA and CDCA and their respective glycol-, tauro- and sulpho-derivatives. The total secondary BAs is the sum of LCA and DCA and their respective glycol-, tauro- and sulpho-derivatives, as well as hyodeoxycholic acid (HDCA, which differs from DCA in that the 6 $\alpha$ -hydroxyl group is the 12 position) and its tauro-derivate. Results are presented as means  $\pm$  standard deviations. *p* values of <0.05 were considered as statistically significant.

### **4.3 Results**

#### **Demographic Data**

Demographics of the 30 patients (15 BAD: 15 IBS-D) are seen in table 18.

**Table 18: Demographic data of the study participants**

	<b>BAD (n=15)</b>	<b>IBS-D (n=15)</b>
<b>Age (years)</b>	53.1 ± 16.3	42.5 ± 17.4
<b>Gender</b>	11/15 (73.3%) F 4/15 (26.7%) M	11/15 (73.3%) F 4/15 (26.7%) M
<b>Ethnicity</b>	15/15 (100%) Caucasian	14/15 (93.3%) Caucasian 1/15 (6.7%) Indian
<b>BMI</b>	28.3 ± 4.6	30.2 ± 8.7
<b>SeHCAT value (%)</b>	6 ± 3.0	33 ± 18.6
<b>Severity of BAD</b>	1/15 (6.6%) Mild 7/15 (46.7%) Moderate 7/15 (46.7%) Severe	N/A
<b>Type of BAD</b>	2/15 (13.4%) Type 1 11/15 (73.3%) Type 2 2/15 (13.4%) Type 3	N/A

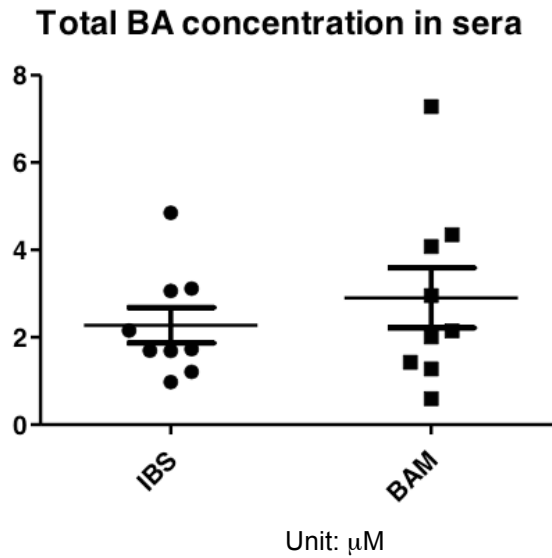
- 24 of the 30 patients (12 with BAD and 12 with IBS-D) had a body mass index (BMI) recorded.
- 2/15 IBS-D patients were diagnosed purely using the Rome III criteria (Rome IV was unavailable at the start of this study) and were not investigated with a SeHCAT study.
- Results expressed as means ± SD.

### ***Bile Acid Composition in Serum***

All results are expressed as means ± the standard error of measurement.

There was a trend towards an increased concentration of total serum BAs in patients with BAD compared to those with IBS-D, with a mean concentration of  $2.90 \pm 2.07 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $2.28 \pm 1.21 \mu\text{mol L}^{-1} \text{g}^{-1}$ , respectively, but this did not reach statistical significance ( $p = 0.73$ ) (figure 16).

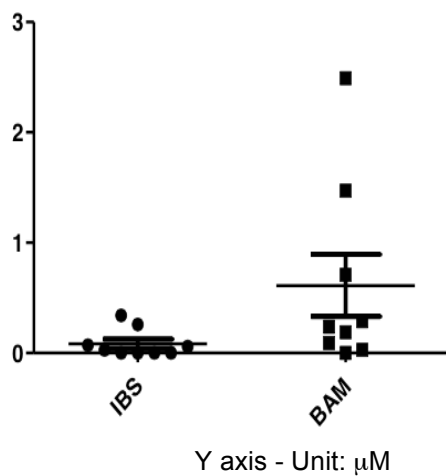
**Figure 16: Total serum BA concentrations**



The concentration of free (unconjugated) CDCA (against total concentration of serum BAs) was statistically higher in patients with BAD compared to those with IBS-D, with a mean concentration of  $0.61 \pm 0.84 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $0.08 \pm 0.13 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.05$ ), respectively (figure 17). The percentage of free CDCA was also statistically higher in patients with BAD compared to those with IBS-D, with a mean percentage of  $18.6 \pm 20.5\%$  and  $2.76 \pm 4.1\%$  ( $p = 0.02$ ), respectively (figure 18).

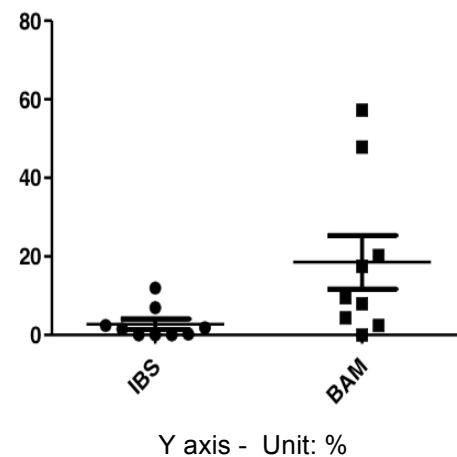
**Figure 17: Concentrations of serum CDCA**

**Free CDCA concentration in sera**



**Figure 18: Proportions of serum CDCA**

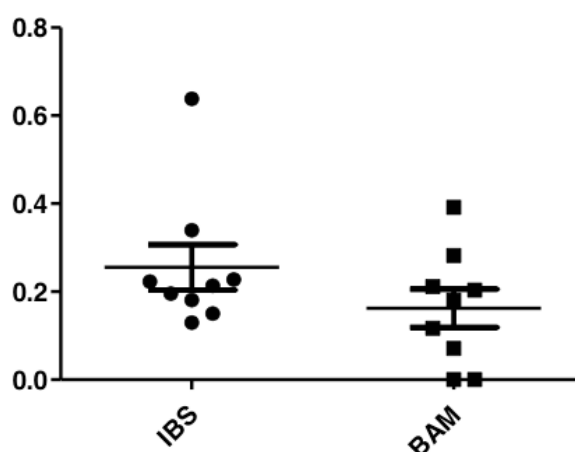
**% Free CDCA in sera**



Concentrations of total sulphated BAs in the serum were similar for both patients with BAD and IBS-D, with mean concentrations of  $0.15 \pm 0.13 \mu\text{mol}$

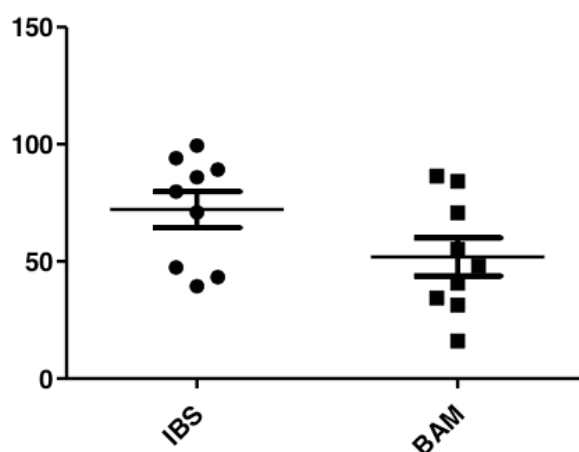
L<sup>-1</sup> g<sup>-1</sup> and  $0.24 \pm 0.15 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.20$ ), respectively (figure 19). There was a marked increase in the percentage of conjugated BAs in patients with IBS-D (mean percentage of  $72.2 \pm 23.1\%$ ) compared to those with BAD (mean percentage of  $51.9 \pm 24.4\%$ ) but this did not reach statistical significance ( $p = 0.09$ ) (figure 20).

**Figure 19: Concentrations of total serum sulphated BAs**



Y axis - Unit:  $\mu\text{M}$

**Figure 20: Proportions of serum conjugated BAs**



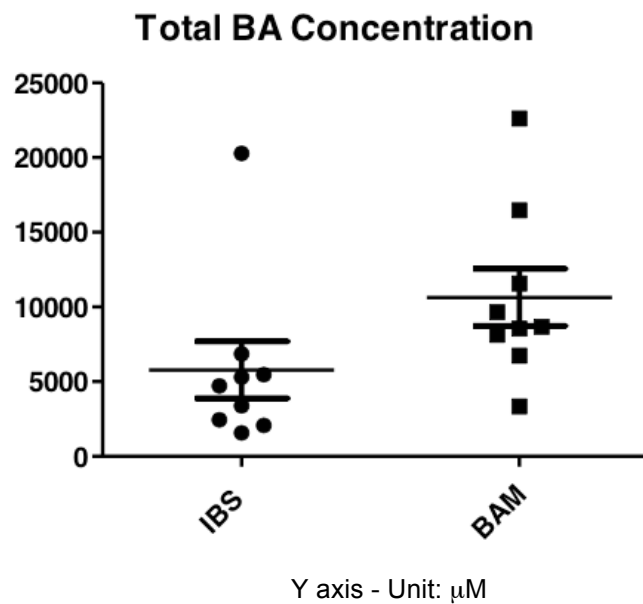
Y axis - Unit: %

### ***Bile Acid Composition in Faeces***

All results are expressed as means  $\pm$  the standard error of measurement.

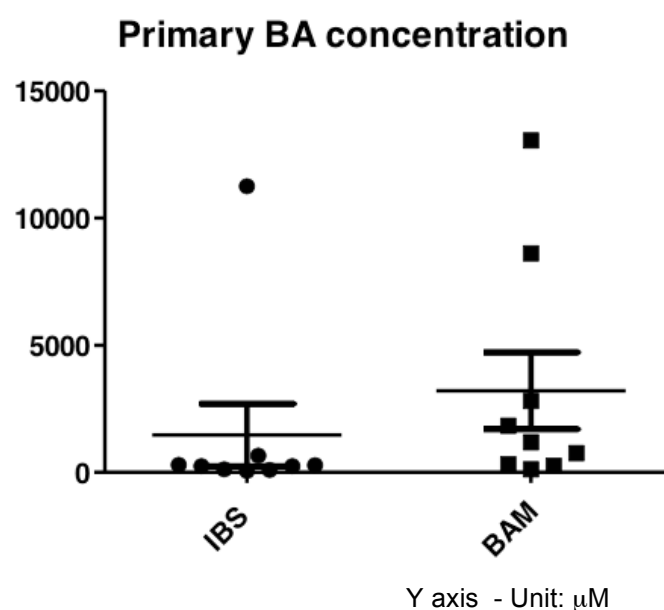
There was a statistically higher concentration of total faecal BAs in patients with BAD compared to those with IBS-D, with a mean concentration of  $10640 \pm 5723 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $5791 \pm 5714 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.02$ ), respectively (figure 21).

**Figure 21: Total faecal BA concentrations**

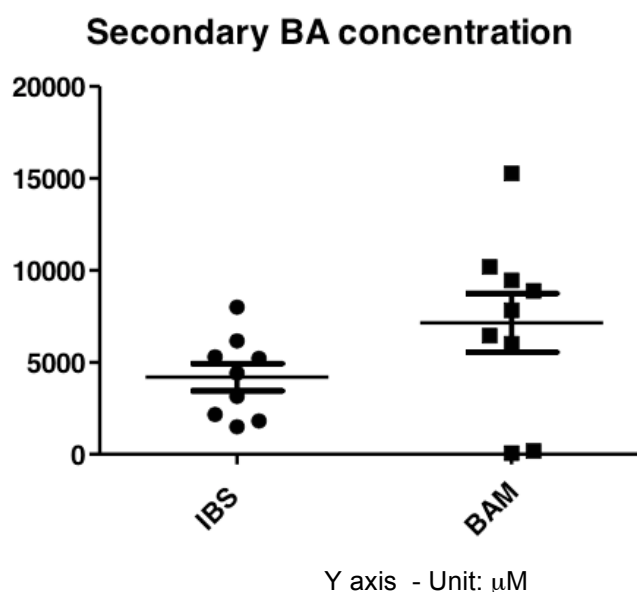


The concentration of primary BAs was statistically higher in patients with BAD compared to those with IBS-D, with a mean concentration of  $3212 \pm 4537 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $1472 \pm 3669 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.05$ ), respectively (figure 22). There was a trend towards an increased concentration of secondary BAs in patients with BAD compared to those with IBS-D, with a mean concentration of  $7139 \pm 4801 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $4184 \pm 2202 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.11$ ), respectively, but this did not reach statistical significance (figure 23). However, the ratio of percentage faecal primary:secondary BAs was markedly increased in patients with BAD (mean percentage of  $17.5 \pm 46.3\%$ ) compared to those with IBS-D (mean percentage of  $0.5 \pm 0.9\%$ ) but this did not reach statistical significance ( $p = 0.30$ ).

**Figure 22: Concentrations of faecal primary BAs**



**Figure 23: Concentrations of faecal secondary BAs**

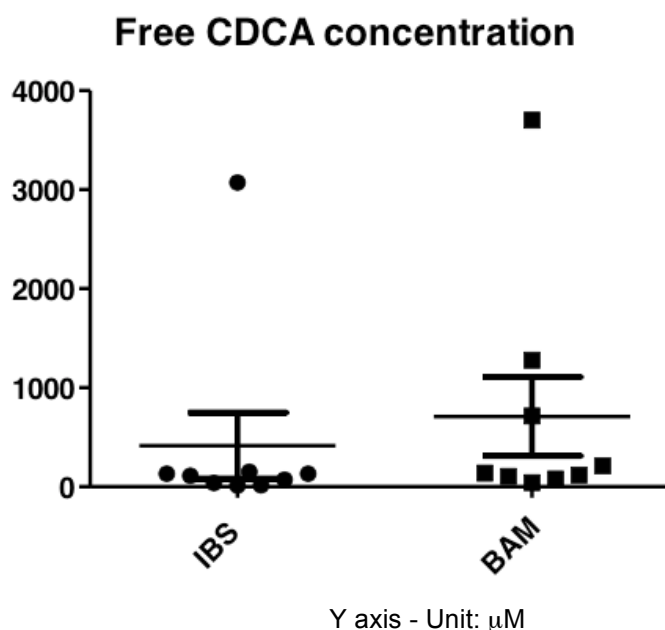


There was a notable trend towards an increased concentration of free (unconjugated) CDCA (similar to serum) in patients with BAD compared to those with IBS-D, with a mean concentration of  $709.7 \pm 1194 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $415.4 \pm 997.7 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.19$ ), respectively, but this did not reach statistical significance (figure 24). There was also a marked trend towards an increased concentration of total DCA in patients with BAD compared to

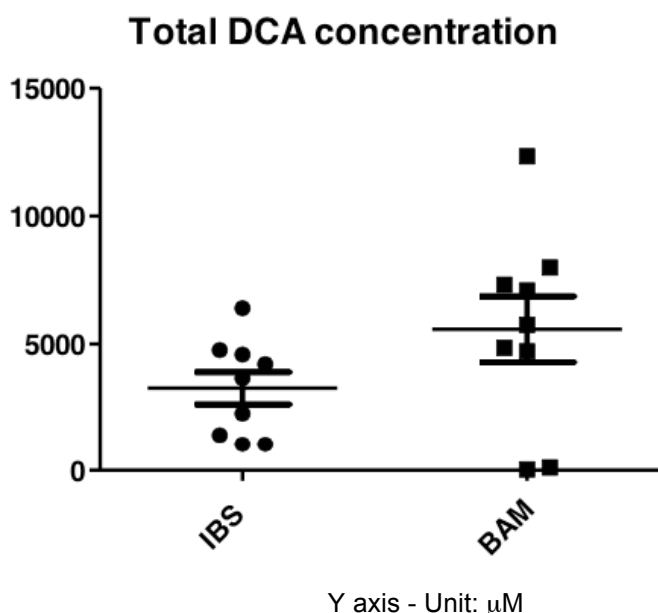


those with IBS-D, with a mean concentration of  $5569 \pm 3848 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $3264 \pm 1905 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.11$ ), respectively, but this did not reach statistical significance (figure 25).

**Figure 24: Concentrations of faecal CDCA**



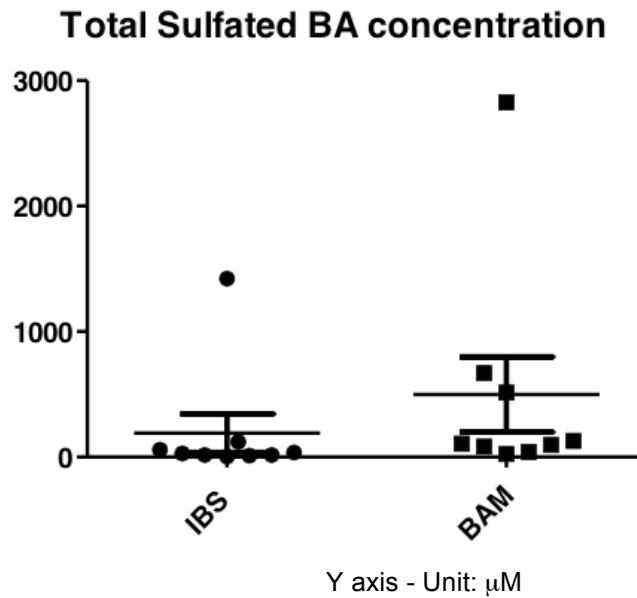
**Figure 25: Concentrations of faecal DCA**



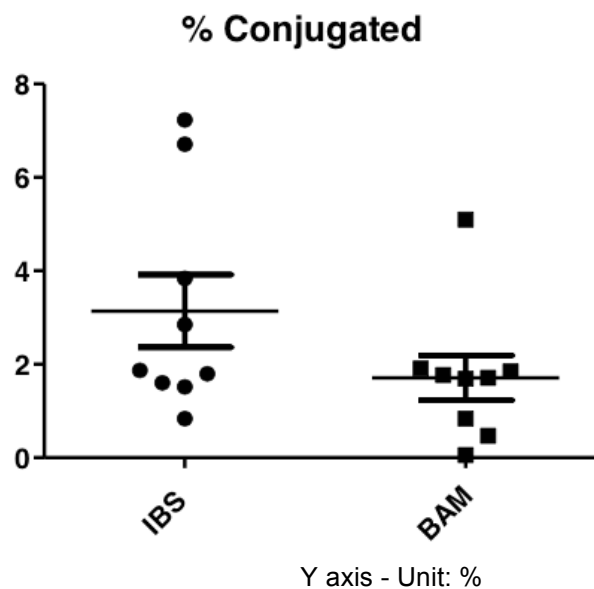
A statistically greater concentration of total sulphated BAs was seen in patients with BAD compared to those with IBS-D, with a mean concentration of  $499 \pm 901.5 \mu\text{mol L}^{-1} \text{g}^{-1}$  and  $190.6 \pm 462.8 \mu\text{mol L}^{-1} \text{g}^{-1}$  ( $p = 0.04$ ), respectively (figure 26). There was a marked increase in the percentage of

conjugated BAs (similar to serum) in patients with IBS-D (mean percentage of  $3.1 \pm 2.3\%$ ) compared to those with BAD (mean percentage of  $1.7 \pm 1.4\%$ ) but this did not reach statistical significance ( $p = 0.20$ ) (figure 27).

**Figure 26: Concentrations of total faecal sulphated BAs**



**Figure 27: Proportions of faecal conjugated BAs**



## 4.4 Discussion

The efficiency of the liver in clearing BAs from the portal circulation, through the presence of the Na<sup>+</sup>-taurocholate co-transporting polypeptide (NTCP) on the basolateral membranes of hepatocytes, maintains low levels of serum concentrations of BAs with new BA synthesis only contributing to a small proportion of the BA pool size(510). The estimated hepatic fractional uptake of CA is 90% and 70-80% for CDCA and DCA(511). The efficiency of ileal resorption of BAs also allows for only small quantities of peripheral BAs with serum BAs increasing if there is reduced hepatic clearance or presence of portosystemic shunting but not by intestinal malabsorption. In patients with primary BAD, who have no intestinal disease, low plasma levels of FGF19 have been observed, resulting in defective negative homeostatic feedback with increased BA synthesis and consequently, the BA pool is enlarged(512).

This is the first study of its kind, which investigates the faecal and serum BA composition in patients with BAD and compares it to that in patients with IBS-D. The composition of the BA pool in both IBS-D and BAD to date has been poorly studied. There is minimal data on the measurement of BAs in IBS-D and none at all in patients with BAD. Duboc's study has observed the total faecal BAs in IBS-D to be similar to those in healthy controls (HCs). In this study, a significant increase in the levels of primary faecal BAs in IBS-D was observed compared to HCs with a corresponding significant decrease in secondary BAs. A reduction in DCA but not LCA was demonstrated. The proportion of faecal primary BAs positively correlated with higher stool frequency and Bristol stool score (lower stool consistency)(157). In a more recent study, the same research group observed increased serum primary BAs in IBS-D and constipation-predominant IBS (IBS-C) patients compared to HCs. However, although reduced levels of serum secondary BAs in IBS-D patients were observed compared to HCs, this was not replicated in IBS-C patients. Faecal BA analysis in IBS-D and IBS-C patients revealed both an increase and parallel decrease in primary and secondary BAs,

respectively, compared to HCs. In patients with IBS-C, this difference with HCs did not reach statistical significance. An increase in faecal sulphated BAs was observed in patients with IBS-D compared to those with IBS-C and HCs(370).

The overall increase in total serum and faecal BAs observed in patients with BAD compared to those with IBS-D is expected as they have an enlarged bile acid pool(513). Serum BA profiles have been found to reflect faecal BA profiles(374). The significant increase in total faecal BAs in the BAD cohort is likely secondary to a combination of reduced ileal absorption in those with ileal disease as well as increased BA delivery to the colon through an overall increased BA pool and impaired negative feedback through reduced FGF19 levels in patients with primary BAD.

The observation of a statistically greater concentration of total faecal sulphated BAs in patients with BAD, with similar serum concentrations in both disease cohorts, is expected given that sulphated BAs are excreted in the faeces and not reabsorbed in the ileum(370). The intestinally expressed enzyme, sulfotransferase-2A1, catalyses the production of sulphated BA analogues, which aids in their detoxification, increases their solubility and excretion in urine and faeces(514). Certainly, it is assumed that increased concentrations of detoxified, sulphated BAs would help to maintain intestinal homeostasis but this effect may be reversed by dysbiosis occurring through BA dysmetabolism in BAD.

A statistically higher concentration of faecal primary BAs and the markedly increased ratio of percentage faecal primary:secondary BAs was observed in patients with BAD. DCA accounts for more than half of the total faecal BAs therefore a relatively reduced proportion of secondary BAs may indicate overall reduced BA transformation activity by the gut microbiota given that secondary BAs are produced exclusively through bacterial enzymatic reactions(59, 371). Compared to conventional mice, germ-free mice demonstrate a discernible increase in primary BAs(374). Secondary BAs in particular, display anti-inflammatory properties through the

mechanism of preferentially activating TGR5, a G-protein-coupled receptor, which reduces the production of pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ )(374). Therefore, a less than expected proportion of secondary BAs compared to the percentage of primary BAs observed in BAD will result in perturbed intestinal homeostasis.

The reduced serum and faecal percentage of conjugated BAs observed in patients with BAD compared to those with IBS-D contradicts the assumed reduced intestinal microbial activity seen otherwise. Dysbiosis with increased numbers of non-deconjugating bacteria may prevail in this cohort, although this remains unknown. Given the laxative properties of BAs, it is plausible that the increased BA pool in patients with BAD may contribute to increased intestinal transit, with consequent reduced time for microbial deconjugation to occur. Most conjugated primary BAs are absorbed in the ileum therefore in patients with BAD who have ileal disease, a reduced serum percentage of conjugated BAs is expected(59).

The concentration and percentage of free (unconjugated) serum CDCA was statistically higher in patients with BAD compared to those with IBS-D. Intestinal bacteria determine the proportion of unconjugated BAs, which are absorbed into the portal venous blood(515). Serum CDCA should therefore reflect the quantity of conjugated and unconjugated CDCA absorbed from the ileum and colon, with the colon being the primary source of unconjugated CDCA.

CDCA is the most potent inducer of FGF19, through FXR agonism(76). Patients with primary BAD have demonstrated low levels of FGF19 and this inversely correlated with BA synthesis as measured by increased serum C4 (7 $\alpha$ -hydroxy-4-cholesten-3-one: a BA precursor and intermediary of BA synthesis) levels(11). With increased BA synthesis, there is associated increased C4 plasma levels as C4 spills over from the hepatocyte into the plasma in direct proportion to the rate of BA synthesis(513). As a result of impaired homeostatic feedback by FGF19, there is a net increase in BA production, evidenced by the raised proportion of serum CDCA, which may

also overspill from the portal into the peripheral circulation. Reduced FXR activation by CDCA as well as its reduced ileal absorption in patients with secondary BAD may result in increased colonic delivery and increased faecal concentration of free (unconjugated) CDCA observed in patients with BAD. This positive association between the raised serum and faecal unconjugated CDCA concentrations results from the CDCA fraction that is absorbed from the colon, which suggests a pathogenic role of CDCA in BAD.

CDCA has been suggested as the cause of diarrhoea in patients with BAD, therefore the increased faecal and serum CDCA observed in this patient group is to be expected(59). Bile acids are known endogenous laxatives and when they exceed concentrations of 3 mmol/L in the colon, physiological changes occur(509). It is assumed that BAs cause secretion by accelerating colonic transit, an assumption supported by the observation of post-cholecystectomy diarrhoea resulting from presumed BA loss(72). DCA has demonstrated consistent secretion at 3mM and CDCA at 5mM concentrations, respectively(516). In human ileal and colonic tissue, CDCA and DCA have exhibited reduced net Na<sup>+</sup> absorption and increased Cl<sup>-</sup> secretion, an effect not observed with CA(35, 37, 517). Rectal CDCA administration can induce colonic propagation waves with oral administration of the same BA, in a dose-dependent manner, accelerating colonic transit and increasing defecation frequency(72, 362). Previously, CDCA was given as treatment for gallstone dissolution but its frequent side effect of diarrhoea made way for UDCA to be utilized instead, given the fact it bears no motility or secretory role in the colon(518).

The role of BAs in promoting fluid and electrolyte secretion through a range of effects in the colon is well documented in animals and cultured epithelia models(518). Mechanisms of action include high concentrations of BAs ( $\geq 1\text{mM}$ ) increasing tight junction permeability, allowing for penetration of the epithelial cells basolateral membrane and increasing free cytosolic Ca<sup>2+</sup>, which is involved in transcellular Cl<sup>-</sup> secretion(519). The role of the intestinal microbiota in modifying BA structure has demonstrated structural specificity

of BA-induced  $\text{Cl}^-$  secretion, with CDCA and DCA stimulating secretion. LCA and various epimers of DCA and CDCA were either inactive or less effective(520). CDCA, but not UDCA, induces dose-dependent  $\text{Cl}^-$  secretion, which is thought to arise from mast cell and histamine-mediated processes and therefore, this is the likely mechanism of increased bowel transit seen in the BAD cohort of patients(521). DCA has exhibited actions on more than one target in the colon to induce secretion: luminal  $\text{K}^+$  channels with an increased cAMP production and  $\text{Cl}^-$  secretion in mucosa and activation of  $\text{Ca}^{2+}$ -regulated indirect  $\text{Cl}^-$  secretion in the crypts(522). Promotion of secretion via varying concentrations of BAs differs significantly between species as well as between BA forms(35, 516).

Opposing actions of BAs at high and low concentrations have been observed. In contrast to the pro-secretory and cytotoxic (decreased transepithelial resistance-TER) effects of BAs at pathophysiological levels, lower physiological levels have demonstrated chronic down-regulation of colonic epithelial secretory function. Anti-secretory effects of chronic (24 hours) exposure to reduced DCA concentrations (10-200 $\mu\text{M}$ ) were observed with inhibited responses to  $\text{Ca}^{2+}$  and cAMP-dependent secretagogues. Other BAs, including CA, CDCA and taurodeoxycholic acid revealed the same effects(516). It has therefore been proposed that the increased  $\text{Cl}^-$  and fluid secretion response to increased BA concentrations, bears a protective function in diluting the luminal contents to avoid epithelial damage as it has been demonstrated that upon removal of DCA, the TER (a measure of epithelial barrier function) was restored(516). Therefore, despite the presence of an enlarged BA pool in BAD, there has been no data to suggest any colonic histological changes in this disease.

Bile acids have been suggested to act as 'osmosignals' in controlling the fluidity of colonic contents. Normally, low colonic DCA concentrations are anti-secretory to aid promotion of colonic absorption. *In vivo*, BA concentrations increase as absorption progresses and luminal contents become dehydrated and when a threshold is breached, DCA performs a

pro-secretory role in rehydrating the luminal contents, therefore preventing the cytotoxic actions exerted by BAs(516). The raised faecal concentration of DCA observed in the BAD cohort may therefore demonstrate a pathophysiological role in this disease.

Due to an improving knowledge of the regulatory role of BAs in intestinal fluid and electrolyte transport, the potential for targeting the FXR in treating diarrhoeal disease has been investigated. In vivo mouse models have demonstrated the anti-secretory effects of the FXR agonists, GW4064 and obeticholic acid (OCA), in the colon. The underlying mechanism resulted from direct inhibition of various components of the epithelial Cl<sup>-</sup> secretory pathway including diminution of both basolateral Na<sup>+</sup>/K<sup>+</sup> ATPase activity and apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channel currents. This in turn, inhibits fluid secretion by preventing Cl<sup>-</sup> uptake across the basolateral membrane and its exit across the apical membrane via CFTR(523).

## **4.5 Conclusions**

It is likely that dysbiosis accounts for the raised faecal primary, sulphated BAs (with loss of this protective mechanism) observed in patients with BAD compared to those with IBS-D (this is further discussed in chapter 6). In addition, the raised concentrations of serum free (unconjugated) CDCA and faecal DCA observed in the BAD cohort may indicate the underlying secretory mechanisms of increased intestinal transit. Serum free (unconjugated) CDCA may play a putative role as a surrogate biological marker of BAD, rather than subjecting patients to investigation with a SeHCAT scan. This would be a simpler test (only a single blood sample required), which would be more widely available, less expensive and time consuming as well as negating the need for repeated hospital visits.

In order to pursue further diagnostic and therapeutic avenues, additional work with a larger population of patients is required to validate this study's



findings and strengthen the relationship observed between bile acids, the intestinal microbiota and the diarrhoeal pathophysiology. Specifically, more detailed molecular studies of microbial enzymatic reactions and consequent BA modification are fundamental to the understanding of the true diversity of bile acid-modifying bacteria and their aetiological role in chronic gastrointestinal diseases. This may also enable establishment of a relationship between numbers and functionality of these bacteria and the risk of development of disease.

# **Chapter 5:**

## **Measurement of**

### **VOCs in BAD and**

### **IBS-D**

## 5.1 Introduction and Aims

### 5.1.1 Introduction

Both BAD and IBS-D present with chronic diarrhoea and it is challenging to differentiate between the two on clinical grounds alone. The SeHCAT (<sup>75</sup>selenium homotaurocholic acid) scan is the gold standard diagnostic modality of choice in confirming a diagnosis of BAD however it is underutilized due to its prohibitive cost (approximately £210 per patient)(54). Thus, there is an unmet clinical requirement for a more widely accessible diagnostic test.

Often, patients with gastrointestinal conditions report an abnormal and unfavourable faecal odour and therefore the 'scent' or composition of faecal gases (volatile organic compounds – VOCs) may be considered as a diagnostic biomarker(475). VOCs are carbon-based chemicals and are classified by their boiling point (ranging from 50 to 260°C) and retention time(435). They are the resultant gas by-products of colonic fermentation by indigenous gut bacteria deriving energy through oxidation of organic compounds (non starch polysaccharides), thereby reflecting bacterial metabolic activity and composition(238). It is assumed that the production of these gaseous products contributes to intestinal homeostasis, through regulating intestinal microbial balance via quorum sensing(238). VOCs are present in exhaled breath and all biological fluids including faeces, urine, blood and sweat(435).

Given that intestinal bacteria are shared by most adults and remains relatively stable, it is presumed that the composition of VOCs is also shared in health with changes occurring in disease. Certainly in acute gastroenteritis, the microbial signature is altered by the pathogen often eclipsing the normal microbiota(457). Rapid intestinal transit has also demonstrated a disturbance in the natural habitat of anaerobes, creating an unfavourable environment for their growth(524). A reduction in the total number of faecal VOCs in diarrhoeal conditions (including ulcerative colitis

and infectious diarrhoea) has been observed compared to healthy donors, suggesting reduced overall biodiversity of the gut flora and decreased synthesis of compounds due to increased intestinal transit time(457).

The measurement of VOCs in urine is possible because of disrupted intestinal permeability(438). Patients with IBS-D have demonstrated increased small bowel and colonic mucosal permeability with reduced gene and protein expression of tight junction (TJ) components of jejunal mucosa(144, 153). Bile acids themselves are considered to disrupt mucosal barrier function through various mechanisms. These include direct epithelial toxicity through their 'detergent' effect on the phospholipid bilayer of the cell membrane, apoptotic effects in reducing ATP production through uncoupling of oxidative phosphorylation and increasing ATP-ase activity as well as by increased bile acid hydrophobicity, concentration-dependent disruption of the intestinal membrane with increasing permeability and changes in TJ proteins mediated by epithelial growth-factor receptor(338).

### **5.1.2 Aims**

In the quest to locate a simple, rapid and reliable biomarker which may be more broadly utilized, we proposed the use of real-time instruments to detect VOCs emanating from biological material to differentiate between IBS-D, BAD and healthy controls (HCs).

## **5.2 Materials and Methods**

### **5.2.1 Recruitment and Sample Collection**

#### ***Ethical Approval***

Patients were recruited as part of the FAMISHED (Food and Fermentation using Metagenomics in Health and Disease) study. Scientific and ethical approval was acquired from the local Research and Development Office as well as Warwickshire Ethical committee ref: 09/H1211/38. Written informed consent was obtained from all participants in the study.

### ***Study Participants***

Patients from the Nuclear Medicine department at University Hospitals Coventry and Warwickshire (UHCW NHS Trust) were recruited from May 2016 to May 2017 after being referred for a SeHCAT scan via attending a gastroenterology clinic with chronic diarrhoea. 26 patients with Rome III criteria IBS-D, 13 patients with BAD and 13 healthy controls (HCs) participated in the study.

Demographic and clinical data including age, gender, ethnicity, BMI, C-reactive protein (CRP – marker of inflammation) and faecal calprotectin levels (a measurement of the protein calprotectin in the stool, which is elevated during intestinal inflammation therefore used to ensure patients with IBD are in remission) for patients with type 1 BAD and co-existing IBD, SeHCAT result, severity and type of BAD were collected.

### ***Sample Collection***

Urine samples were collected in standard specimen collection bottles in the nuclear medicine department and stored at -80°C within two hours of collection. They were transferred to the University of Warwick in dry ice (-78°C) within a polybox and then stored at -80°C.

### ***Inclusion and Exclusion Criteria***

To be included in this cross-sectional study, both male and female participants were required to meet the following criteria: (1) have chronic diarrhoea, (2) diagnosis of BAD based on a SeHCAT retention value of  $\leq 15\%$ , (3) patients with IBD (inflammatory bowel disease) may only be included if type 1 BAD is present, (4) patients who have had a previous cholecystectomy may only be included if type 3 BAD is present.

Participants were excluded from the study if they suffered from coeliac disease, IBD (if type 1 BAD is not present), active IBD (defined as FCP  $> 50$  mg/kg stool or CRP  $> 11$  mg/L at the time of the SeHCAT scan), colorectal

cancer or had been on antibiotics/probiotics in the last three months.

### **5.2.2 Urine Samples**

Urine samples with a shelf life of 12 months or less were only used as our previous work has demonstrated that the concentration and stability of urinary VOCs decreases over time, with optimal storage time being defined as no longer than 12 months. We observed less variation with greater uniformity and tighter clustering of the total number of urinary VOCs released in samples that were stored within 9 months(473).

For this study, urine was the biological sample of choice given our experience of it being the most 'patient and clinician user friendly', when compared with breath, blood and faeces. This is likely due to its non-invasive nature and allowance for being more easily attainable in a clinical setting as well as its availability in large volumes.

### **5.2.3 FAIMS (Field Asymmetric Ion Mobility Spectrometry)**

All urine samples were analysed using a commercial Owlstone Lonestar FAIMS instrument (Cambridge, UK). FAIMS (ion-mobility spectrometry – IMS) allows for gas molecules to be separated and analysed at room temperature and atmospheric pressure(54). It separates complex mixtures by measuring the mobility of ionised molecules in high electric fields. In our case, the vapour (or headspace) from a sample is first ionised with a radiation source (Ni-63 in our case) and passed between two parallel conductive plates. On these plates is applied an alternating electric field, which is created by applying a voltage across the plates(525). This field either attracts or repels and does not affect the ions as they pass between the plates. Any ions that touch the plates lose their charge and exit without being detected. To remove this movement, a fixed compensation voltage is added to the existing electric field. By scanning through a range of electric field strengths and compensation voltages, the instrument is able to produce a mobility map on a sample. To identify underlying trends within the ion

mobility data, pattern recognition techniques are employed and this can be correlated to the presence or absence of a disease process(526).

Prior to testing, the samples were put in the fridge at 4°C to thaw overnight. Just before testing, 5mls of the sample was aliquoted into standard 10ml glass vials (Thames Restek, UK). The glass vial was then placed inside an ATLAS sampling system (Owlstone, UK) and heated to 40°C for 10 minutes. This enables production of a reasonable headspace of volatiles. Then, a flow of dry, clean air was passed over the sample at a rate of 500 ml/minute. An additional 1500 ml/minute was added to this sample air to make a total flow rate of 2 L/minute. The Lonestar unit is set up to cycle through a dispersion field of 0 to 100% in 51 steps (dispersion field is a measure of the electric field strength) and a compensation voltage from +6V and -6V in 512 steps. To ensure the baseline response was returned, blanks of clean, dry air were run before and after each urine sample.

#### **5.2.4 Statistical Analysis**

The data was analysed using a previously developed processing pipeline. Each sample run generates 52,224 data points in a 2D matrix of compensation voltages and dispersion fields. The data was first decomposed using 2D discrete wavelet transform to extract subtle chemical signal (a wavelet is a form of data compression used for audio and visual applications). This also allows for the diffuse 'peaks' observed in the data to be preferentially extracted and correspond to different sets of chemical species. The signal is therefore concentrated in a relatively small number of wavelet coefficients to simplify and improve subsequent analysis steps.

To identify features with discriminatory power, a 10-fold cross-validation was applied, using 90% of the data as a training set and 10% as a test set. Within each fold, a feature selection step was conducted to identify which features in the training set were best to predict disease. Feature selection was performed using the Wilcoxon rank sum test or Kruskal-Wallis test by calculating *p*-values between features and using only the features with the

lowest  $p$ -values (15 features). These data were then used as an input for the PCA (principal component analysis).

Five different classifiers were used for prediction: Sparse Logistic Regression, Gaussian Process Classifier, Neural Network, Random Forest and Support Vector Machine. Of these, the Gaussian Process Classifier and Support Vector Machine produced the best classification results and predicted probability of a sample being either from a HC or a patient with IBS-D or BAD. From these analyses, receiver operator curves (ROC), area under curve (AUC), sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated.

## **5.3 Results**

### **Demographic Data**

Demographics of the 52 patients (13 BAD: 26 IBS-D: 13 HCs) are seen in table 19.



**Table 19: Demographic data of the study participants**

	<b>BAD (n=13)</b>	<b>IBS-D (n=26)</b>	<b>HCs (n=13)</b>
<b>Age (years)</b>	47.8 ± 14.6	43.0 ± 13.6	34.5 ± 9.3
<b>Gender</b>	6/13 (46.2%) F 7/13 (53.8%) M	21/26 (80.8%) F 5/26 (19.2%) M	8/13 (61.5%) F 5/13 (38.5%) M
<b>Ethnicity</b>	12/13 (92.3%) Caucasian 1/13 (7.7%) South Asian	23/26 (88.5%) Caucasian 2/26 (7.7%) South Asian 1/26 (3.8%) Afro-Caribbean	8/13 (61.5%) Caucasian 3/13 (23.1%) Indian 2/13 (15.4%) Afro-Caribbean
<b>BMI</b>	30.0 ± 7.2	28.5 ± 7.0	N/A
<b>SeHCAT value (%)</b>	5 ± 0.0	36 ± 0.1	N/A
<b>Severity of BAD</b>	1/13 (6.7%) Mild 4/13 (30.8%) Moderate 8/13 (61.5%) Severe	N/A	N/A
<b>Type of BAD</b>	1/13 (7.7%) Type 1 9/13 (69.2%) Type 2 3/13 (23.1%) Type 3	N/A	N/A

- 35 of the 52 patients (12 with BAD, 23 with IBS-D, 0 HCs) had a body mass index (BMI) recorded.
- Results expressed as means ± SD.

The BAD cohort of patients (6 female and 7 male) aged 26 to 69 years had a mean BMI of 30.0, defining them as being overweight. The mean SeHCAT value was 5%, which is consistent with severe BAD. Of the 13 patients, 1 (6.7%) had mild, 4 (30.8%) had moderate and 8 (61.5%) had severe

severity of disease respectively. Most (9 – 69.2%) patients had type 2 BAD, 1 (7.7%) patients had type 1 and 3 (23.1%) had type 3 BAD.

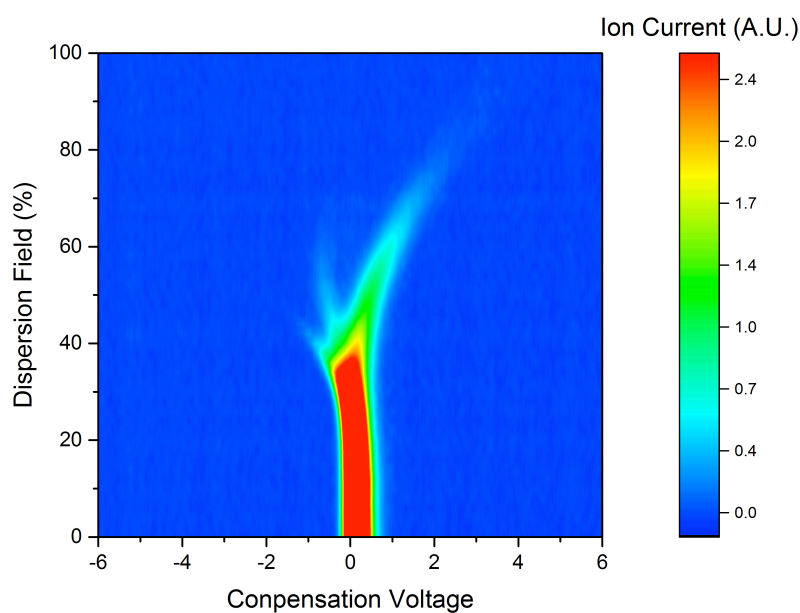
The IBS-D cohort of patients (21 female and 5 male) aged 20 to 66 years had a mean BMI of 28.5, defining them as being overweight. The mean SeHCAT value was 36%, which excluded them from having BAD.

The HCs (8 female and 5 male) were aged 24 to 54 years.

### FAIMS Data

Figure 28 demonstrates a typical FAIMS ‘plume’ output ion scan, where the change in total ion current was used as a feature for data processing.

**Figure 28: Raw data from the FAIMS instrument to an IBS patient urine sample**

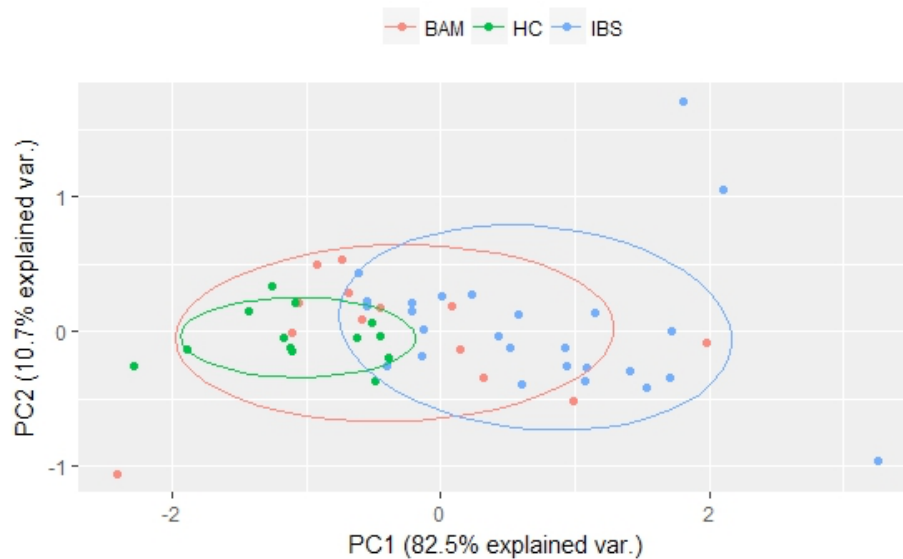


Log of raw data from the FAIMS instrument where intensity is in arbitrary units of ion count.

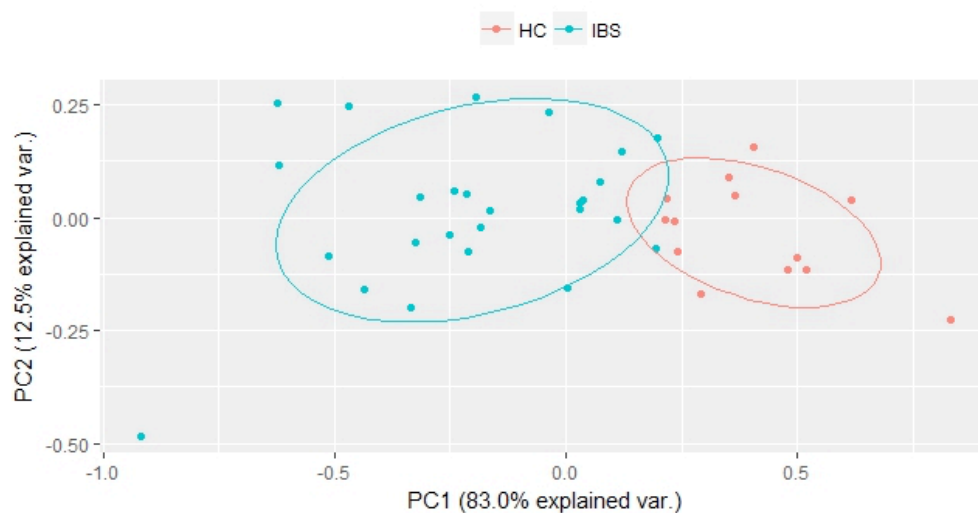
Figure 29a shows the PCA (non-classified technique) with no observable differences between the 3 groups. However, there is separation evident (sensitivity 81%, specificity 100%) between the IBS-D and HC groups (figure 29b) and between the BAD and HC groups (sensitivity 100%,

specificity 38%) (figures 29c and 30). The separation between the IBS-D and HC groups is more distinct compared to the other groups (table 20, figure 31). Less clear separation is observed between the BAD and IBS-D groups (sensitivity 50%, specificity 92%) (figure 29d, figure 32). Therefore for the IBS vs HC data (figure 29b), PCA analysis has most successfully demonstrated linear combinations of the different VOCs that separate out the different clusters (patient cohorts). PCA analysis aims to reduce high dimensional data to demonstrate similarities and differences between the data more clearly. The PC1 axis spans the most variation in the data with the PC2 axis spanning the second most variation in the data.

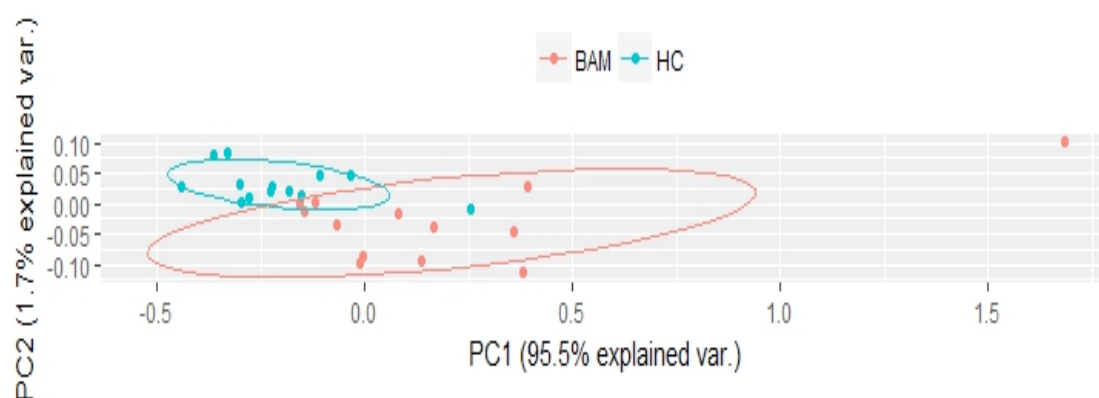
**Figure 29a: PCA plot of the three groups: BAD, IBS and HC**



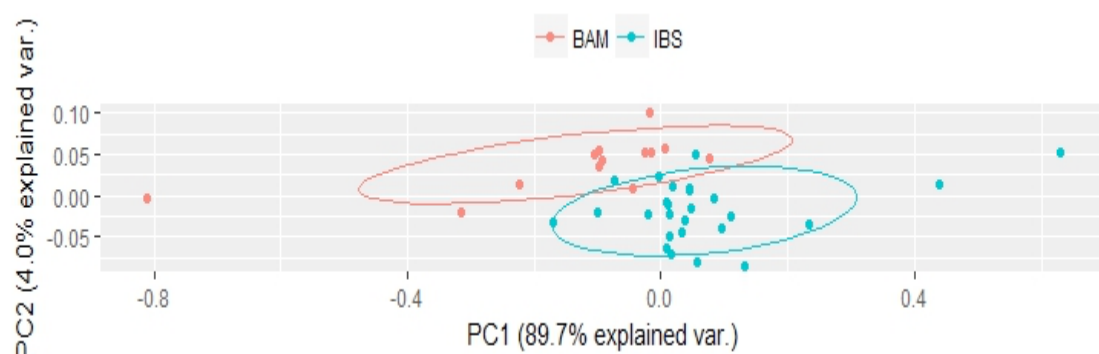
**Figure 29b: PCA plot of IBS and HC**



**Figure 29c: PCA plot of BAD and HC**



**Figure 29d: PCA plot of BAD and IBS**



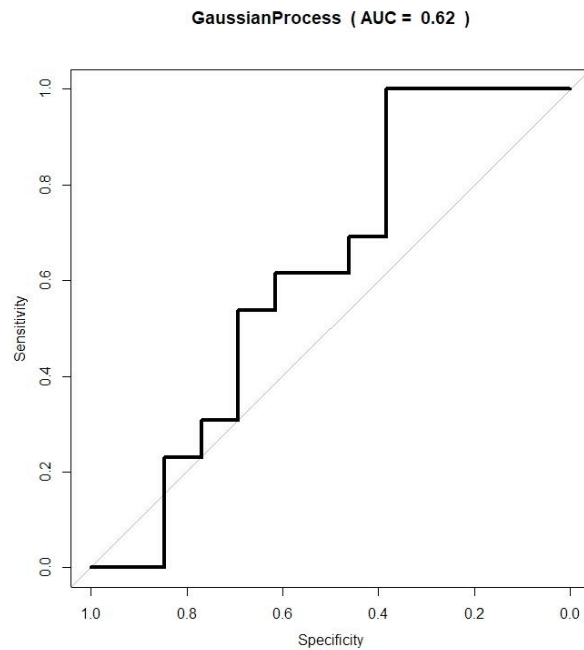
**Table 20: Comparison of FAIMS diagnostic accuracy in differentiating between the different patient groups.**

	<b>IBS vs BAD</b>	<b>IBS vs HC</b>	<b>BAD vs HC</b>
<b>AUC</b>	0.6 (0.42 – 0.78)	0.92 (0.84 – 1)	0.62 (0.38 – 0.85)
<b>Sensitivity</b>	0.5 (0.3 – 0.7)	0.81 (0.61 – 0.93)	1 (0.75 – 1)
<b>Specificity</b>	0.92 (0.64 - 1)	1 (0.75 – 1)	0.38 (0.14 – 0.68)
<b>PPV</b>	0.93	1	0.62
<b>NPV</b>	0.48	0.72	1

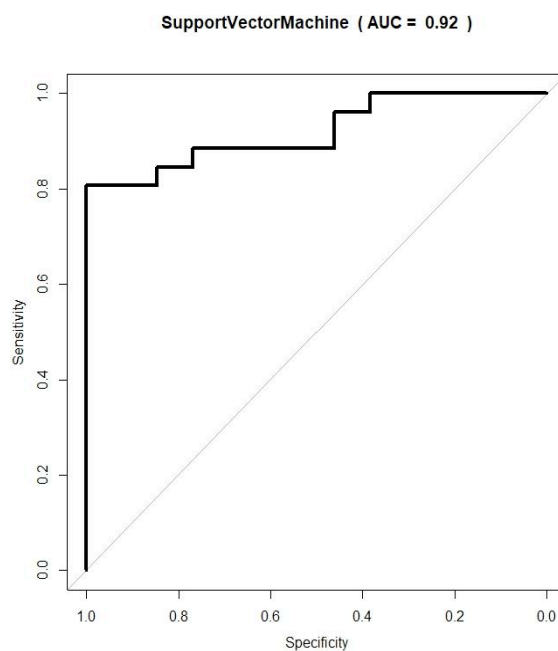
- AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value; (95% confidence interval)

The ROC (receiver operating characteristic) curves below demonstrate relative trade-offs between true positive and false positive results with the best possible prediction method depicting a point in the upper left corner of the ROC space.

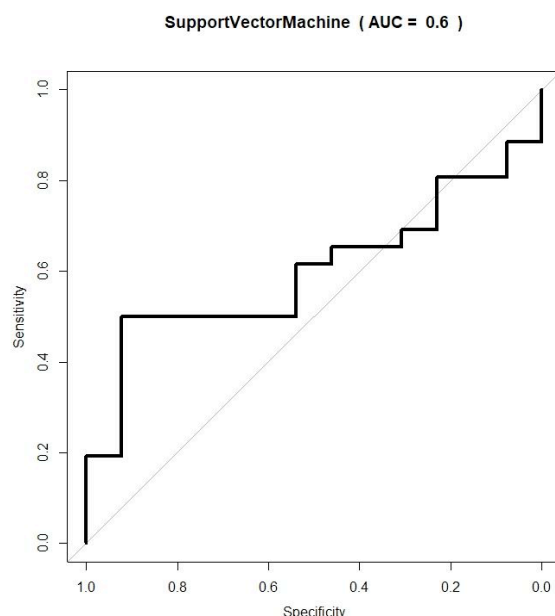
**Figure 30: ROC curve – BAD vs HC**



**Figure 31: ROC curve – IBS vs HC**



**Figure 32: ROC curve – IBS vs BAD**



## 5.4 Discussion

This study demonstrates the utility of a non-invasive IMS technology in differentiating between the main potential causes of chronic diarrhoea and its ability in separating these conditions from healthy controls. This is reflected by the unique VOC profiles of each of these groups. The sensitivity of the FAIMS instrument was greatest in differentiating IBS from HCs and BAD from HCs. The altered gas profile observed in patients with IBS and BAD highlights the important concept of intestinal dysbiosis, where pathological imbalances in the gut microbiota precipitates disease. Therefore, the presence of the VOC chemical footprints observed are the resultant effects of modified intestinal microbiota fermentation.

The IMS technology demonstrated much greater sensitivity in differentiating BAD (100%) and IBS-D (81%) from HCs than between the two disease groups (50%). This suggests that the technology is more precise in recognising abnormal biological and metabolic processes occurring in disease, that differ to those in healthy subjects and therefore distinction between health and disease is clearer than between two disease states.

Certainly VOCs, which vary between patient groups, are thought to be reflective of cellular metabolism either leading to or being indicative of a diseased state(527). Low-grade intestinal inflammation is thought to play an aetiological role in IBS with significant increases in lamina propria immune cells being demonstrated in the colonic mucosa of these patients compared to healthy subjects. More specifically, immune activation, with increased density of T lymphocytes in the mucosa of IBS patients has been observed(528). There is no data to suggest inflammation plays a role in BAD and so it appears that activation of the immune system is the signal picked up the FAIMS instrument. On the contrary, BAs have consistently demonstrated anti-inflammatory properties through NF- $\kappa$ B inhibition and thus decreasing the synthesis of pro-inflammatory cytokines, such as TNF- $\alpha$  in monocytes and macrophages(374). The changes in the VOC profiles observed (greater separation of the IBS-D and HC groups compared to BAD and IBS-D as well as BAD and HCs) may represent this inflammatory pathogenic component to disease. This is further supported by previous work conducted by our research team where FAIMS was able to distinguish between active disease and remission in patients with inflammatory bowel disease(438).

Faecal VOC analysis has reported distinct VOC patterns, which were able to distinguish between patients with IBS-D, active inflammatory bowel disease and HCs. In IBS-D, the volatile cyclohexanecarboxylic acid and its ester derivatives, as well as short chain fatty acids were found to be more abundant compared to the other groups(447). Breath analysis identified a combination of 16 VOCs in discriminating patients with IBS from HCs, which correlated with IBS symptoms(474). The use of FAIMS in patients with IBS-D and coeliac disease has been observed to differentiate between the two disease entities based on their urinary VOC profiles(448). The only study to date in patients with BAD identified clear separation of this cohort based on their urinary VOC profile to HCs and patients with ulcerative colitis. In addition, two chemical peaks, corresponding to acetamide and 2-propanol, were observed in patients with BAD and were either absent or present in

significantly reduced quantities in the HCs and UC cohorts(54). Current research into the VOC profiles in patients with IBS-D and BAD is significantly limited with discordance in the methodologies employed due to heterogeneity in the type of biological sample collected and analytic technique used as well as small sample sizes, therefore clinically relevant inferences from the available data cannot be made.

A particular strength of our study is the achievement of optimal stability of the urine samples. The stability of VOCs is crucial in medical diagnostics in ensuring detection of all available chemical compounds. Coupled with this is the requirement of optimal storage conditions however there is a lack of a standardized protocol for urine sample collection, storage and analysis which requires further work before clinical implementation.

Limitations to the use of urinary VOCs as biomarkers of disease include the influence of multifactorial, uncontrolled variables such as sex, age, ethnicity, diurnal and daily variation, dietary and fluid intake. VOCs are derived from both endogenous and exogenous sources, including flavouring agents, foods and personal care products therefore a degree of inter-individual variation is expected(529). The impact of the intestinal microbiota on the 'fermentome' (a term used to describe the complex relationship between diet, the gut microbiota and VOCs) also remains unknown(238).

It is unclear whether the differing expressions of disease-associated VOCs are a direct consequence or a secondary effect of disease therefore before these can be used as potential biomarkers, the underlying biochemical pathways and mechanisms of origin must be elucidated. VOCs may be produced locally at the site of primary disease through the effects of inflammation, disturbed cell metabolism and cell death. They may also originate more systemically by downstream processes of disease including increased catabolism, immune activation and oxidative stress(525). In disease, new VOCs may be generated, which the body does not normally produce during normal physiological circumstances and/or there is a change in the concentrations of VOCs already present.



In this study, we used sensor technology linked with pattern recognition methods to identify the VOC profile signature in the different disease groups. Unfortunately, this does not allow for the identification of particular chemical compounds, which gas chromatography-mass spectrometry (GC-MS) equipment is equipped to do so but this benefit is outweighed by its high cost. However, identifying an overall pattern of VOCs as a rapid diagnostic tool is likely to be more clinically useful in a busy outpatients clinic and is also transferrable to the primary care setting.

The challenge of FAIMS to accurately and reproducibly detect biomarkers of disease occurring at low concentrations in a complex background of 'noise' continues with method development paralleling the rapid rate of clinical requirement for this technology. This technology lends itself to be programmed fairly easily through the sensor selectively focusing on disease-associated compounds after the initial biomarker validation steps(525). Advantages to employing FAIMS include its portability to clinical settings, cost-effectiveness compared to more expensive endoscopic procedures/radiological tests in the investigation of patients presenting with chronic diarrhoea, attainment of rapid results with real-time and point-of-care diagnosis and its reliability given its high sensitivity and reproducible detector(525).

## **5.5 Conclusions**

In conclusion, there are discernible differences in the VOC signatures of the three patient groups. Our study is limited by the small sample size and we would expect the trends observed from our data to be magnified with increasing study power. Nevertheless, we have been able to demonstrate the utility of the FAIMS in characterizing disease-specific VOC profiles through associated biological effects reflective of underlying gut bacterial fermentable activity for both BAD and IBS-D. It is plausible that inflammatory processes may either trigger or be a result of changes in the gut microbiota, which is demonstrated in the VOC profiles observed. In

place of the SeHCAT scan, VOCs may be used to confirm a diagnosis of IBS-D and exclude BAD given its stronger ability in differentiating patients with IBS-D from HCs rather than those with BAD from HCs. These preliminary findings may help to develop a point of care diagnosis based on FAIMS technology, which would certainly be more favourable to the patient, inexpensive and less time-consuming as well as being more practical and clinically applicable compared to bacterial culturing and metagenomic techniques.

# **Chapter 6:**

# **Discussion**

## **6.0 Discussion**

### **6.1 Introduction and Rationale for Study**

During recent years, increasing evidence has supported human health being greatly influenced by the gut microbiota and for this reason, the intestinal microbiome is often symbolized as the ‘forgotten organ’ due to its extensive metabolic activity and abundance of genes impacting host metabolic pathways(530, 531). The recognised synthetic (e.g. production of vitamins B and K), metabolic (e.g. fermentation and absorption of indigestible carbohydrates) and immune (e.g. maintenance of intestinal barrier function, maturation and development of cell-mediated and innate immunity) functions of the gut microbiome maintain intestinal homeostasis and health(530).

Historically, individual microbes have been related to pathogenic infection and a specific disease state however more recently, the concept of dysbiosis with a pathological imbalance in a microbial community has been recognised to explain complex phenotypes of disease, including IBD and IBS(157, 374, 532). In relation to this, dysmetabolism of BAs has been intricately linked to intestinal dysbiosis. Evidence suggests this is secondary to a reduction in bacteria bearing BSH activity, which is central to BA deconjugation and maintenance of normal BA metabolites and BA receptor signaling required to regulate intestinal homeostasis via the TGR5 and FXR receptors(530). Therefore, it is imperative that the composition and metabolic functionality of the intestinal microbiota and its relationship with BA metabolism must be closely examined to appreciate its implications in health and disease.

rRNA gene amplicon sequencing allows us to profile the gut microbiota with the 16s rRNA gene representing the target of choice for bacterial ecological studies and taxon identification(533). However, this technique is restricted in providing information of how the microbiome dynamically interacts with the

host to influence health. Therefore beyond metagenomics, the use of metabolomics is vital in measuring bacterial metabolites and providing an insight into metabolic and biochemical pathways as a means of assessing bacterial function. Bacterial metabolites, including BAs, SCFAs and VOCs are influenced by dietary substrates, which in turn may alter the metabolomic profile, yielding variable effects on the host. The new, modified host physiology and phenotype can consequently have a feedback effect on the indigenous gut microbiota(531).

The chronic diarrhoeal gastrointestinal diseases, BAD and IBS-D, share similar clinical presentations with most clinicians repeatedly missing the opportunity to diagnose BAD. This is further compounded by the fact that the gold standard diagnostic test (SeHCAT scan) for BAD is limited in its availability. Despite the fact that neither of these conditions predispose patients to severe illness, targeting treatment to the correct diagnosis is essential in improving quality of life as well as reducing significant economic costs through work absenteeism and patients frequently attending hospital for diagnostic tests and medical consultations.

The aim of this thesis was to investigate the differences in intestinal microbial communities between the BAD and IBS-D patient groups and through study of the microbial metabolites produced, interrogate the underlying cellular mechanisms by which the microbes and metabolites may contribute towards the aetiology of these conditions. Given that the metabolome is influenced by the gut microbiome (as well as environmental and genetic factors), the microbial metabolites produced by the microbiome may perform as potential biomarkers of the underlying microbial composition and disease state.

## **6.2 Summary and Implication of Results**

### *a) Link between Intestinal Microbiome and SCFAs*

A significant difference in bacterial diversity was observed between the BAD and IBS-D patient cohorts. Reduced diversity and an abundance of

anaerobic taxa, including including *Bifidobacteria*, *Prevotella*, *Lachnospiraceae*, *Verrucomicrobia* and *Bacteroides* were observed in the BAD group compared to patients with IBS-D. SCFAs are end products of anaerobic microbial metabolism and therefore this may account for the significant increase in the total concentration of SCFAs demonstrated in the BAD cohort compared to those with IBS-D(252). A bidirectional relationship where SCFAs shape the composition of the microbiota may also be true as a significant inverse relationship between total SCFA concentrations and faecal pH value has been observed with a reduction in pH enhancing populations of butyrate-producing bacteria and curtailing the growth of *Bacteroides*(240, 534). Therefore, the increased SCFA concentration in the BAD cohort may have caused the intestinal dysbiosis observed in this group or vice-versa.

The concept of cross-feeding, where one gut commensal utilizes the end products of metabolism or the energy rich complex carbohydrate breakdown products from another commensal, has a significant impact on the overall production of SCFAs(493). This may explain the dysbiosis observed and proliferation of certain groups of bacteria in the disease cohorts. Some *Bifidobacterium* species are unable to use inulin-type fructans but can grow in the presence of inulin degraders by using the mono- and oligosaccharides released by these bacteria(535). Therefore outgrowths of taxa such as this, as was the case in the BAD cohort, may be explained by symbiotic relationships existing between groups of bacteria.

The above mutualistic relationship existing between bacteria may also account for the significant increase in the concentrations of acetate and propionate demonstrated in the BAD cohort compared to those with IBS-D. There was also a significant increase in the concentration of propionate in BAD compared to HCs. It is recognised that bacterial cross-feeding mainly occurs from acetate to butyrate and to a lesser extent between butyrate and propionate but almost negligibly between propionate and acetate(493). An animal study demonstrated utilization of acetate produced by a species of *Bacteroides* (this genus was abundant in both BAD and IBS-D) by

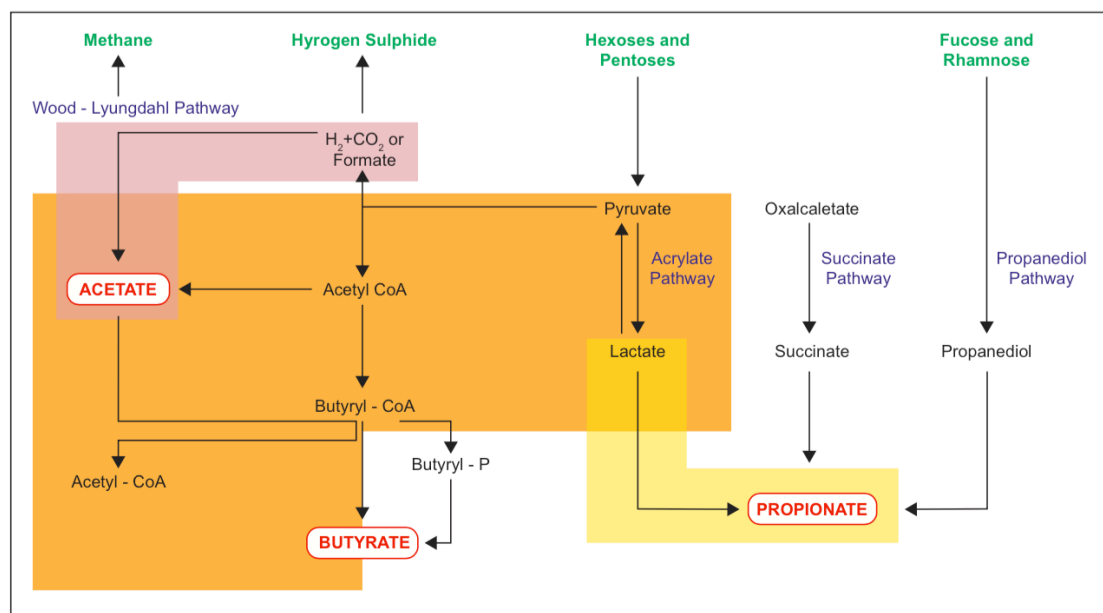
*Faecalibacterium prausnitzii*(536). Therefore, intestinal dysbiosis with specific *Bacteroides* species may alter metabolic fluxes between commensals, preventing normal symbiotic relationships from occurring, which may result in unexpected proportions of individual SCFAs.

Figure 33 depicts the metabolic pathways responsible for the biosynthesis of the three major SCFAs. Acetate production via the microbiota arises from two main metabolic routes. The majority arises from microbial fermentation of carbohydrates. A third arises from acetogenic bacteria, which through the Wood-Ljungdahl pathway, synthesise acetate from hydrogen and carbon dioxide or formic acid(493). Acetogenic bacteria belong to a large, diverse group of facultative (organisms able to survive in both aerobic and anaerobic habitats) and obligate (species for which oxygen is toxic) anaerobes. These include *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Staphylococcus*, *Escherichia coli*, *Pseudomonas* and *Streptococcus*(537). The BAD cohort demonstrated an abundance of *Bifidobacterium* compared to the IBS-D group, which may have contributed to the increased concentration of acetate observed.

Three pathways contribute to the formation of propionate with the majority being mostly formed via the succinate pathway by *Bacteroidetes* (abundant in both the BAD and IBS-D cohorts) and some Firmicutes belonging to the *Negativicutes* class(488). The proportion of propionate present against the total faecal SCFA correlates with the relative abundance of *Bacteroidetes*(538). Despite the abundance of the *Bacteroides* genus of bacteria seen in both BAD and IBS-D, it may be hypothesised that particular propionate-producing species within the *Bacteroides* taxa are more abundant in the BAD cohort. It is recognised that an increased input of BAs results in a significant inhibition of the *Bacteroidetes*(377). Therefore, it is plausible that through excess BAs seen in BAD, certain species of the *Bacteroides* taxa are selectively knocked out, leaving behind ones that are capable of specifically producing propionate, which may be a protective mechanism towards their survival in an otherwise 'toxic' environment as BAs display antimicrobial properties in high concentrations(378).

The acrylate pathway, using lactate as a substrate in its conversion to propionate, is limited to a few members of the *Lachnospiraceae* and *Veillonellaceae* families. The third route of propionate production, the propanediol pathway, is characterized by conversion of deoxy-sugars to propionate and involves Proteobacteria and members of the *Lachnospiraceae* family(493). Surprisingly, an abundance of the *Lachnospiraceae* genus group of bacteria was observed in the IBS-D cohort compared to the BAD group, which suggests that the succinate pathway may be the main route for propionate production in BAD, given the significantly increased concentration of propionate in these patients compared to those with IBS-D. Also, through competition for nutritional resources, an abundance of certain bacterial species that are not involved in propionate production may be associated with inhibition of other species that are required for propionate production. It is therefore likely that through dysbiosis, the differences in microbial composition are accompanied by alterations in microbial metabolites that define these bacteria.

**Figure 33: The metabolic pathways responsible for the biosynthesis of the three major SCFAs: acetate, butyrate and propionate.**



The shaded geometric shapes represent the cross-feeding mechanisms for the production of the three main SCFAs: acetate, butyrate and propionate



### *b) Link between Intestinal Microbiota and Bile Acids*

The difference in the BA profiles between the BAD and IBS-D cohorts is expected given that a difference in bacterial diversity between the two groups has been demonstrated. The statistically significant increased concentration of primary BAs observed in BAD compared to those with IBS-D is likely due to the reduced bacterial diversity seen in BAD with consequent reduced bacterial biotransformation activity in the conversion of primary to secondary BAs. In parallel to this, dysbiosis was detected in the faeces of BAD patients. The taxonomic group of *Ruminococcus* bacteria, which was found to be more abundant in IBS-D, contains many species with 7 $\alpha$ -dehydroxylating activity, crucial to the formation of secondary BAs(378). Therefore, a reduced quantity of these bacteria in BAD compared to those with IBS-D would lead to the increase in faecal primary BAs and primary to secondary BA ratio observed.

As a result of dysbiosis, with reduced bacterial diversity and reduced biotransformation activity demonstrated in BAD, an increase in primary BAs such as CDCA is expected. The increase in free (unconjugated) serum and faecal CDCA in BAD is an interesting finding as it suggests that despite the dysbiosis observed in these patients, deconjugating activity via BSH-producing bacteria is unaffected. However, the widespread distribution of BSHs across Gram-positive and negative intestinal bacteria with multiple isoforms of the enzyme existing in certain strains, does not permit an understanding of the mechanisms by which BSHs enable bacteria to colonise the colon(371). BA modification is therefore a widespread property of the intestinal microbiota however the data suggests that in BAD, dysbiosis is likely to affect the final stage in the pathway of BA modification. Despite functioning BSH-deconjugating activity, it is the final step of BA 7 $\alpha$ -dehydroxylation reactions, which is impaired and results in the retention of the primary BA, CDCA in the enterohepatic circulation, over spilling into the systemic circulation. This is likely to be a crucial factor in the mechanism of developing BAD.

An increase in faecal sulphated BAs in BAD was observed. The liver is the predominant site of BA sulphation and this process is considered to decrease BA toxicity and enhance BA elimination therefore, this finding may be an adaptive change to alleviate BA accumulation in BAD and a protective mechanism in the setting of intestinal dysbiosis(514).

Altogether, these results suggest that BAD patients exhibit defective BA metabolism through intestinal dysbiosis.

*c) Link between Intestinal Microbiota, VOCs and SCFAs.*

The greatest separation of VOC profiles exist between the IBS-D and HC cohorts, followed by less clear separation between the BAD and HC groups with further minimal separation between the BAD and IBS-D groups. This supports the notion of VOCs reflecting the fermentation processes of underlying intestinal microbiota with evidence of dysbiosis in both disease cohorts (although this is greatest in BAD with overall reduced bacterial diversity) therefore, it is difficult to separate out these two group, which are both alike with respect to altered fermentation profiles. The separation of groups is therefore more apparent when comparing a diseased or abnormal (BAD or IBS-D) fermentation profile to a healthy one (HCs).

On the other hand, the greatest differences in SCFA production were found between the BAD and HC cohort, rather than the IBS-D and HC groups, therefore it is assumed that fermentation is more altered in the former comparison of groups, which would suggest greater separation in their respective VOC profiles although this was not found to be the case. Therefore, it would appear that production of these metabolic by-products did not mirror altered fermentation. A possible explanation for this is that there were no observable differences in the branched SCFA levels between the IBS-D and HC groups however levels of these were mostly significantly lower in the BAD group compared to HCs. Little is known about microbial-derived branched SCFAs, where protein fermentation occurs. The amino acid substrates may be utilized by the colonic bacteria to produce SCFAs, ammonia and other BCFAs(502). Therefore, with reduced

substrates/precursors being supplied to the intestinal microbiota, there may be an overall reduced production of metabolic end-products, resulting in a reduced VOC 'signal'.

### **6.3 Concluding Remarks and Future Directions**

The findings of these experiments collectively contribute to the existing body of scientific literature in IBS-D, where dysbiosis has been demonstrated in the presence of BA dysmetabolism and altered levels of SCFAs have been observed. It also parallels other VOC studies, which have discriminated IBS VOC profiles from other disease groups and HCs. However, the experiments within this thesis are the first in bringing new findings in 16s rRNA gene amplicon sequencing and metabolomics in BAD, which have not been previously studied and suggests alternative mechanisms to the aetiology of this disease. Moreover, this thesis measures and evaluates in turn, the role of the gut microbiome, SCFA and BA production to elucidate their intricate relationship in the pathophysiology of IBS-D and BAD, which no previous study has collectively achieved. Through these thesis's studies, it is proposed that functional output of the microbiota, rather than abundance of specific genera, is the main modulator of disease.

The main limitation of these experiments is cohort sample size, which impacted on the power of the studies. Further study with a larger cohort of samples is required to investigate if the trends observed in these studies can be replicated and reach statistical significance. Although there was some overlap in the patients used for each study within this thesis, utilising the same patient cohort consistently would confirm the relationship between the gut microbiome, SCFA and BA production and the resultant translation to differing VOC profiles.

The 16S rRNA sequencing technique employed, analysed bacteria at the genus taxonomic level, which meant that some genera were found to be abundant in both disease cohorts as information on individual species was

unavailable. Although species identification may have improved characterization of the microbiome in the disease cohorts, BA metabolism and production of SCFAs occur via overlapping groups of bacteria, therefore assessment of specific bacterial species may not have provided further answers with regards to overall bacterial functionality and genesis of pathology. In addition to this, the reliability of species definition being based on 16S rRNA sequence similarity clustering, as well as the resolution of the 16S rRNA gene being too low at times to allow the differentiation of closely related species, is a limiting factor(533). Developments in bacterial genomics and species interaction will enhance knowledge of bacterial community ecology and delineate the microbiome in further detail, facilitating understanding of new aetiological mechanisms underlying BAD such as specifically identifying bacteria involved in the proteolysis degradation of branched SCFAs. This may merit further study to assess whether the resultant impaired amino acid metabolism from dysbiosis affects FXR-mediated BA signalling causing dyslipidaemia and BAD. Our studies were not designed to answer these questions but further insights into the pathological role of the gut microbiota and the functioning role of its resultant metabolites would not only provide a platform to search for non-invasive diagnostic biomarkers but also lead to the development of novel therapeutic targets.

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## **Appendix**

1. Patient information sheet
2. Patient consent form

## **FaMIsHED Study**

### **A study to look at how bowel fermentation and diet affect other diseases**

#### **PATIENT INFORMATION SHEET (PIS) – for Gastroenterology patients**

##### **1. Invitation**

You are being invited to take part in a research study. Before you decide if you want to take part, you may wish to consider two things: firstly, why the research is being done and secondly what you would have to do. Please take time to read the information below and talk it over with someone else if you want to. If anything is not clear or you would like to know more, please ask.

##### **2. What is the reason for the study?**

Bowel problems are common and diet is known to be important in certain diseases of the large bowel (colon). Within the bowel there are large numbers of bacteria which help in the process of fermentation. Changes in diet may result in changes in the bacteria or fermentation which is thought to be contributory not only to certain bowel diseases but other metabolic diseases such as diabetes and obesity and even kidney, bladder, joint and heart disease. We hope that this study will give us a better understanding of the relationship between diet and bowel fermentation and its effects on other diseases.

##### **3. Why have I been chosen?**

Your consultant has invited you to participate in this research because you are having tests for, or have been diagnosed with a condition which is the subject of this research study. You may also have been asked to take part if you do not have a specific illness to act as a comparison with other people with different conditions or diseases.

##### **4. Do I have to take part?**

It is for you to decide whether you wish to take part. If you do decide to take part you will be given this information sheet to keep. You will be asked to sign a consent form. Even if you decide to take part, you can withdraw at any time. If you decide to withdraw, you do not need to tell us why and it will not change your treatment in any way.

##### **5. What will happen to me if I take part?**

If you agree to take part, we will invite you to sign a consent form to participate. You may be eligible to donate some of the following samples- urine, stool, blood, breath, sputum, rectal mucosal swab or tissue biopsy. We will also ask your permission to look at your medical records, including your medical history and medication.

Depending on the medical condition you are having tests for (or) if you are a healthy volunteer, we will collect certain samples e.g. urine, blood, and breath or bowel tissue. However, not all may be relevant to you. A member of the research team will explain in detail which samples we would like to collect from you. Most samples will be collected at your hospital appointment, but you may be asked to collect urine & stool samples at home and either post it or bring it with you to the hospital at your next appointment or drop off at GP. The research team will tell you if you need to do this and give you the necessary forms and specimen pots.

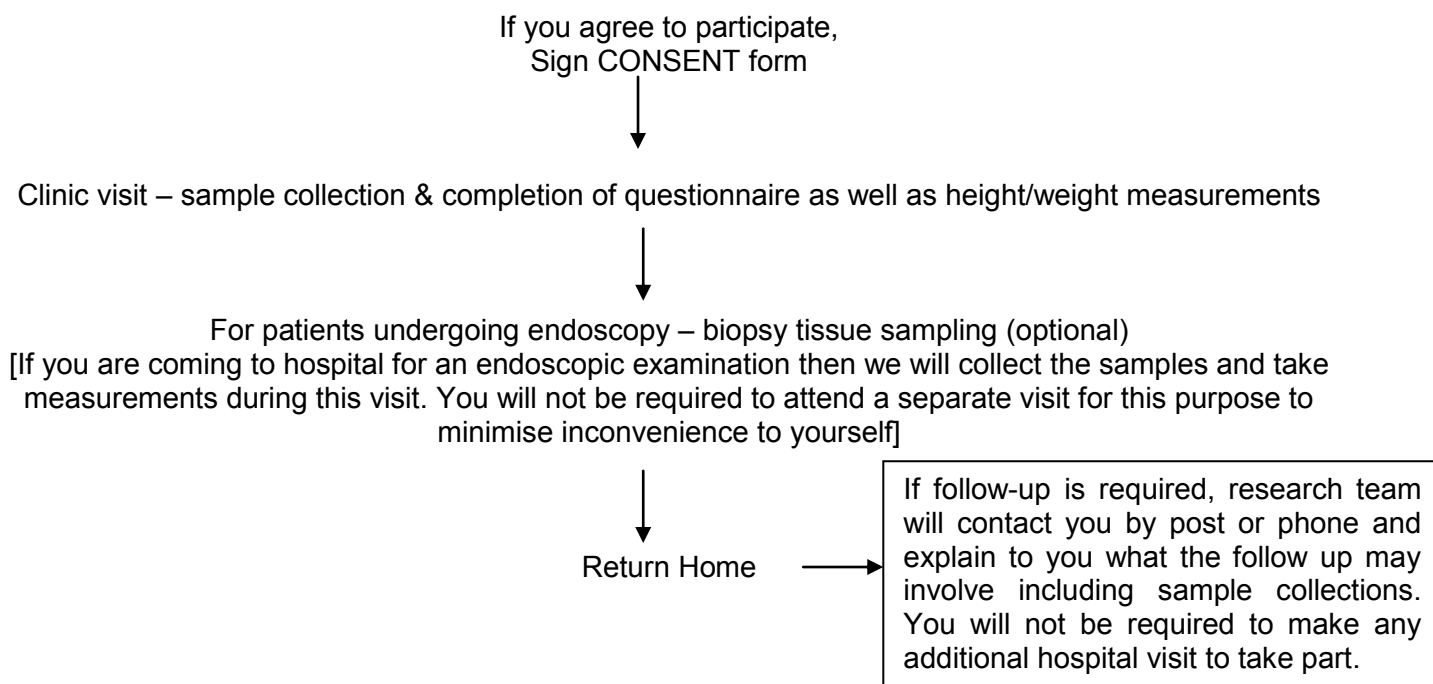
##### *For those attending Endoscopy*

If you are due to have an endoscopic procedure (including OGD, sigmoidoscopy or colonoscopy) as part of your medical care, we will ask your permission to take a tissue biopsy from you during the procedure). The

doctor doing the test will take pieces of tissue for examination under a microscope (this is known as taking a biopsy). An extra, 2 to 4 small pieces of tissue will be taken (each being the size of a grain of rice) for this study. Taking these extra biopsies will not affect your care or add any significant risk. Your endoscopic procedure will be the **same** whether or not you take part in the study. The doctor will discuss the results of your test either during or after the examination.

Depending on the condition you are having tests for, you may be asked to complete a food and lifestyle questionnaire which should take about 20 min. This can often be completed during your hospital visit. If this is not possible, the form can be completed at home and posted back to us. Recordings of your height, weight, hip and waist circumference will also be made (if not already done on your clinic visit).

After this, you can return home. If you attend follow-up clinic as per your routine treatment, we may ask you again for further samples (e.g. urine, stool or blood). You will not be required to make an additional visit to the hospital for this purpose. Below is a chart of what will happen.



### C. Difficile – optional sub-study

We are also looking specifically into *Clostridium difficile*, also known as *C. difficile* or *C. diff* and this is a bacteria that can infect the bowel and cause diarrhoea. This infection most commonly affects people who have recently been treated with antibiotics. We want to be able to stratify risk of the ongoing disease and potentially guide therapy in this area. In order to do this we may ask your permission to take samples via an instrument which will be inserted directly into your rectum – this may be done anyway as part of your clinical assessment standard care. The procedure is known as *mucosal sampling* and is not dangerous. It is merely to sample the lining of the bowel. You do not have to take part if you do not wish. You will be asked to complete consent to this part of the study separately if you agree to take part.

## **6. What do I have to do?**

You don't need to do anything until you come into the hospital for an appointment. At this point, if you agree to participate, one of the the research team will discuss this with you and obtain your consent. If you have any questions beforehand, please speak with us (details below).

## **7. What are the possible disadvantages and risks of taking part?**

- Bleeding very rarely occurs following biopsies and the risk of bleeding is small. (In one study, there was one case of bleeding noted after 5000 consecutive biopsies i.e. 0.02% risk). Our own experience in a previous study had no immediate complications following 4500 consecutive biopsies in 500 individuals
- Taking a blood sample can be a little uncomfortable and occasionally for some there is a little bruising.

- c) The additional biopsies required will add only 3-5 minutes to the procedure.
- d) In certain selected cases where an endoscopy is not planned we may ask for your permission to take samples via an instrument inserted into the rectum which is also referred to as *mucosal sampling*. This procedure is to assess the lining of the bowel and is not dangerous but may be potentially uncomfortable and may be part of your routine care.
- e) It is hoped that all necessary information can be obtained during the clinic or endoscopic visit and if not this will add no more than 20 min to your overall visit.

**8. What happens to the samples taken?**

Samples collected as part of this study will be stored for analysis. We will also request permission to store samples for future research studies. This is because in the future, newer techniques may become available which will allow us to perform more up to date testing. All of your samples will be stored anonymously which means that the results of the analysis cannot be traced directly back to you. However, significant clinical findings which relate directly to your care will be fed back to your overseeing consultant and to your GP.

**9. What are the possible benefits of taking part?**

Whilst taking part may not be particularly beneficial to you, we hope that the information we get from this study will help us understand more about how diet affects certain bacteria, genes and proteins in the normal bowel. In the future this may help us understand more about how certain diseases develop and perhaps even how to better prevent this through alteration in diet.

**10. Will my taking part in this study be kept confidential?**

All the information we have about you from this study is strictly confidential. This information will be kept securely while the study is taking place and only members of the research team will be able to access your information. They may also be looked at by representatives of regulatory authorities and by authorised people to check that the study is being carried out correctly. Any information about you that leaves the hospital will have your name, address and any other personal information removed so that you cannot be recognised. Your GP and your hospital consultant will be told that you are taking part in the study if you wish.

**11. What if something goes wrong?**

In the unlikely event that something goes wrong, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service (NHS) complaints mechanisms will be available to you.

**12. Who is organising and funding the research?**

This study has been organised by UHCW NHS Trust in conjunction with the University of Warwick. The chief investigator is Professor R P Arasaradnam. None of the researchers will be paid in person nor will they receive any financial gain for doing this study. Funding in the form of Research Grants have been obtained from local charities, research networks and collaborative bids (commercial and non-commercial).

**13. Who has reviewed the study?**

The West Midlands – Coventry and Warwickshire Research Ethics Committee has reviewed and approved the study

**14. Contact for Further Information or Complaints**

Local Contact names and telephone numbers:

Research Nurse.: Ms. Subie Wurie, Ms. Leighanne Burns, Ms. Kirstie James

Tel no.....: 02476967724

FaMIsHED Study IRAS: 18717

Contact details.....: Research & Development  
ADE30003, 3rd Floor Opposite Ward 30

PALS (Patient Advice and Liaison Service) on freephone 0800 028 4203 or email: [Feedback@uhcw.nhs.uk](mailto:Feedback@uhcw.nhs.uk)

Study Chief Investigator:  
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Tel: 02476 966087  
Email: [ramesh.arasaradnam@uhcw.nhs.uk](mailto:ramesh.arasaradnam@uhcw.nhs.uk)

Thank you for taking part in the study.

## CONSENT FORM: FaMIsHED Study

Study Title: Food and fermentation using metagenomics in health and disease	Participant Study No: Participant Details:
Chief Investigator. Prof Ramesh Arasaradnam	
Site:	

No	Statement	Please <b>ONLY INITIAL</b> (not tick)	
1	I confirm that I have read the Participant Information Sheet (PIS) V8.0 dated 08.02.2017 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.		
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.		
3	I agree to give the following samples for this study which will be stored for analysis (or) for future research studies	<b>INITIAL</b> yes (or) no <b>YES</b> <b>NO</b>	
	a) Blood		
	a) Urine		
	b) Stool		
	c) Breath		
	d) Sputum		
	e) Rectal mucosal swab		
	f) Tissue biopsy		
4	Any procedures for obtaining samples and the related risks, if any, have been explained to me and I understand that this is for research purposes only.		
5	I agree that my samples may be used by collaborators (academic or commercial) by application to the Arden Tissue Bank and my personal details will not be shared in any form or manner.		
6	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.		
7	I understand that the information collected about me will be used to		

	support research studies in the future, and may be shared anonymously with other researchers.	
8	I agree to being contacted by a member of the research team by telephone or letter at a later date and that I am willing to answer questions related to symptoms and my general health.	
9	I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.	
10	I agree to my General Practitioner (GP) being informed of my participation in this study.	
11	I agree to take part in the above study.	

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Name of Participant

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Date

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Signature

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Name of Person Taking Consent

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Date

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Signature

***When completed: 1 copy for the participant; 1 in their medical notes, and keep the original in the study site file.***